

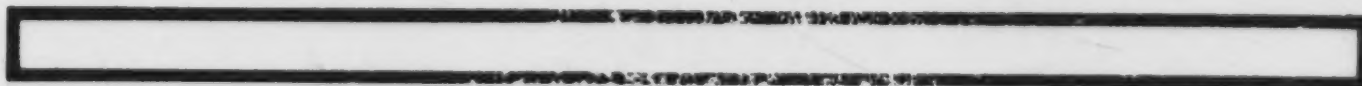
Quantitation of Viruses in
Waste and Other Waters

Syracuse Univ., NY

Prepared for

Environmental Monitoring and Support
Lab.-Cincinnati, OH

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QUANTITATION OF VIRUSES
IN WASTE AND OTHER WATERS

by

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Grant No. R-803774

Project Officer

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FOREWORD

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Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The Environmental Monitoring and Support Laboratory - Cincinnati conducts research to:

- o Develop and evaluate methods to measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid wastes.
- o Investigate methods for the concentration, recovery, and identification of viruses, bacteria, and other microbiological organisms in water; and, to determine the responses of aquatic organisms to water quality.
- o Develop and operate an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.
- o Develop and operate a computerized system for instrument automation leading to improved data collection, analysis, and quality control.

This study was designed to gain insight into the binding of viruses to sludge solids, the factors responsible for their binding, and how each of these factors contribute to the production of persistent populations of infective viruses in treatment plant effluents and sludge-bed runoffs. Such information is important because of the need to develop a sound data base from which to assess the methodology for the monitoring of water-borne viruses.

Robert L. Booth

Robert L. Booth, Acting Director
Environmental Monitoring and Support
Laboratory - Cincinnati

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ABSTRACT

During treatment of domestic sewage by the activated sludge process large numbers of enteroviruses and other water-borne viruses are removed, principally by adsorption to the microbial floc. The numbers of viruses which are removed by adsorption vary over a wide range due to largely unknown factors. This study describes the interactions between the virions and exopolysaccharide surfaces of gram negative, urealytic floc bacteria and demonstrates adsorption of virus particles by extracellular polyhydroxybutyrate granules produced by Zoogloea ramigera under carbohydrate shock loads.

Factors which affect the virus-exopolysaccharide interactions include the types of organisms, presence of heavy metals and multivalent cations, bulking conditions of the sludge treatment, pH and humic acid or other organic compounds. Some virions are released during wastage of the sludge flocs and the presence of exopolysaccharide antigens on the surface of the free, infectious virus particles can be detected.

In the laboratory, viruses can be transferred to protozoa which graze on the flocs in aerobic treatment processes and on the suspended solids in effluent. This study examines the fate of both free and adsorbed virions after wastage of the floc. Purified entero-, reo-, adeno-, and SV40 viruses were labeled with ^{125}I by lactoperoxidase or chloroglycoluril methods. They were adsorbed to flocs of ultraviolet-irradiated bacteria (strains of Zoogloea ramigera, Enterobacter aerogens, and nonpigmented pseudomonads from sludge) and fed to ciliates. The zoomicrobes were centrifuged or filtered to remove bacteria and their virus content was determined as ^{125}I equivalents or PFU. Appreciable quantities of viruses were acquired by several ciliates in axenic cultures and by mixed ciliates which were obtained by flotation from primary sludge. The ^{125}I -virus counts in the ciliates were inversely proportional to the reduced number of bacteria. Retention of intact virions was determined by using viruses labeled with ^3H -uridine and ^{125}I . Retention times varied with different feeding regimes. Protozoa excreted 90 percent of the ^{125}I in 2 to 72 hours and small numbers of viable poliovirus 1 were excreted. Attempts to transfer ^{125}I -viruses from ciliate to ciliate or to cysts failed.

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SECTIONAL
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CONTENTS

	Page
Foreword.....	iii
Abstract.....	iv
Figures.....	vi
Tables.....	vii
Abbreviations and Symbols.....	x
Acknowledgement.....	xi
 1. Introduction	
Scope and Objectives of the Study.....	1
Background Information.....	1
2. Conclusions.....	6
3. Recommendations.....	8
4. Materials and Methods.....	10
5. Experimental Results	
Adsorption and recovery of viruses from treatment plant sludges.....	19
Adsorption of viruses by specific microbes: bacteria, algae and ciliates.....	30
Interaction of viruses with microbial polysaccharides and PHB granules.....	42
Recovery of adsorbed viruses.....	50
Role of metals and pH in virus adherence to sludge solids.....	50
6. General Discussion and Conclusions.....	56
7. References.....	65

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OF TEXT

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FIGURES

Number

Page

1. Presumptive assay for viruses from activated sludge samples..... 13
2. Identification and quantification of viruses in waste waters
after concentrating them with the Aquella instrument virus
concentrator..... 14

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vi

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SECTIONS
HERE

TABLES

<u>Number</u>		<u>Page</u>
1	Possible Flocculation Reactions for Colloidal Particles Destabilized by a Polyelectrolyte Flocculant.....	4
2	Viruses.....	10
3	<u>Zoogloea ramigera</u>	10
4	Fractionation of Sludge Solids by Differential Centrifugation.....	15
5	Virus Isolates from Treatment Plants.....	21
6	Removal Rate of Poliovirus 1 With Activated Sludge Solids.....	24
7	Association of ¹²⁵ I-labeled Poliovirus 1 With Various Fractions From Raw, Undigested Sludge.....	25
8	Ability of Fresh and Frozen Sewage Solids to Adsorb Poliovirus.....	27
9	Comparison of Virus-containing Sucrose Gradient Profiles of Four Digested Sludge Samples.....	28
10	Reproducibility of Sucrose Density Gradient Fractionation of Virus-Containing Sludge Solids From the Anaerobic Digester Tanks.....	29
11	Adsorption of Radioiodinated Poliovirus 1 by <u>Zoogloea ramigera</u> Cells Grown Six Hours in Low Carbon-Nitrogen Medium.....	32
12	Adsorption of Radioiodinated Poliovirus 1 by Polyhydroxybutyrate Granules.....	33
13	Adsorption of Phage f2 by PHB Granules.....	33

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OF TEXT

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FOR TABLES
AND ILLUSTRATIONS

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BEGIN
SECTION
HERE

Number		Page
14	Adsorption of Radioiodinated Viruses by <u>Zoogloea ramigera</u> Cells Grown Six Hours in Low Carbon-Nitrogen Medium.....	34
15	Adsorption of ¹²⁵ I-poliovirus 1 by <u>Zoogloea ramigera</u> Cell Floes Treated With Metal Ions.....	35
16	Adsorption of ¹²⁵ I-poliovirus to Beads Coated with Pure Cultures of Bacteria From Activated Sludge Floes.....	37
17	Oxygen Dependent Decay of Viruses in MLSS After Seeding With Trivalent Poliovirus Vaccine.....	38
18A	Removal of Radioiodinated, Adsorbed Polio-virus 1 by Ciliated Protozoans 12 hrs After Feeding Bacteria or PHB Granules.....	40
18B	Role of Ciliated Protozoans in the Removal of ¹²⁵ I-Poliovirus Adsorbed to <u>Zoogloea ramigera</u> and/or Its Constituents.....	41
19	Stability of ¹²⁵ I-Poliovirus Removed by <u>Tetrahymena Pyriformis</u> Grazing on <u>Zoogloea Ramigera</u>	43
20	Adsorption of PV-1 by Algal Pollutants Treated With 0.005M Metal Ions.....	44
21	Removal of ¹²⁵ I-poliovirus Adsorbed to Native PHB Granules.....	45
22	Adsorption of ¹²⁵ I-poliovirus 1 by <u>Zoogloea ramigera</u> Exocellular Polymers Treated With Metal Ions.....	47
23	Recovery of ¹²⁵ I-poliovirus 1 From <u>Zoogloea ramigera</u> I-16-M Exocellular Polymer Treated with Various Cations.....	48
24	Adsorption of Poliovirus by Crude Polymer Extracts of Sewage Solids.....	49
25	Desorption and Recovery of Radioiodinated Poliovirus 1 From PHB Granules and <u>Z. ramigera</u>	51
26	Desorption of PFU From Sludge Solids.....	52

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TRATIONS

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BEGIN
SECTIONS
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Number

Page

- 27 Adsorption of ^{125}I -labeled Viruses by Dried
Anaerobic Digester Sludge pH 3.5, 0.005M AlCl_3 54
- 28 Aluminum/pH Interaction on the Surface Film of an
Adherent Pseudomonad, 78-004..... 55
- 29 Comparison of Virus Adsorbing Capacity of
Sludge Bacteria Isolated by Glucose-Induced
Adherence After Growth in Peptone Medium..... 58
- 30 Influence of pH on the Adsorption of ^{125}I -poliovirus 1
by Zoogloea ramigera I-16-M Cell Flocc After Coating
With Aluminum Ion..... 62

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AND ILLUSTRATIONS

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LIST OF ABBREVIATIONS AND SYMBOLS

BBL	- Baltimore Biological Laboratory division of Bioquest, Becton Dickinson Company
BGM	- Buffalo green monkey kidney cells
DLVO	- Derijaguin Landau, Verwey and Overbeek theory of colloidal stability
DPM	- disintegrations per minute
EDTA	- ethylenediaminetetraacetic acid; Versene
EMC virus	- encephalomyocarditis virus
f2	- RNA coliphage f2
g	- gram, gravity
gal	- gallon
HEL	- human embryonic lung cells
HEK	- human embryonic kidney cells
HEp-2	- human epidermal carcinoma cells
IgG	- gamma globulin
L	- liter
L929	- mouse connective tissue cell line
MEM	- Eagle's minimum essential medium for tissue cultures; EMEM, with Earle's basal salts; RMEM, with Hank's basal salts
mg	- milligram
MGD	- million gallons per day
MGG stain	- May-Grunwald-Giemsa stain
mL	- milliliter
nm	- nanometer
PBS	- phosphate buffered saline
PFU	- plaque forming unit
PHB	- polyhydroxybutyrate
RPM	- revolutions per minute
TCID ₅₀	- tissue culture 50 per cent average infective dose (of virus)
SD	- standard deviation
U	- unit (of antibiotic)
Vero	- African green monkey kidney cells
$\bar{x}$	- arithmetic mean
XPS	- exopolysaccharide
ZR	- <u>Zoogloea ramigera</u> --strain

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We are particularly indebted to our EPA Project Officer, Robert S. Safferman, EPA Environmental Monitoring and Support Laboratory, Cincinnati, OH, for his help in obtaining reference materials and cultures, for valuable suggestions regarding the experimental work, and for his great patience when progress reports were overdue.

An expression of our appreciation is due Georgia Ventura, Eileen O'Connor and Betty Smith for skillful preparation of this report.

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SECTION 1

INTRODUCTION

SCOPE AND OBJECTIVES OF THE STUDY

It is apparent from many published reports that enteroviruses and other types of viruses are not entirely removed by conventional primary and secondary sewage treatment even in efficient, properly maintained facilities. Precisely what quantity of virus is removed by these processes has been difficult to establish, but most estimates indicate 1 to 2 log reduction in titer by activated sludge treatment methods (1-4). Primary settling of waste waters removes few virus particles, but it appears that with a small amount of manipulation the clarifiers can be used effectively for tertiary treatment of sewage and can be operated in modes which will also remove viruses (5).

Sludge solids are generated by methods which concentrate viruses effectively without degrading them. Consequently the types and concentration of virus pathogens must be considered in the disposal of the sludges. There also exists the possibility that after activated sludge has concentrated viruses by adsorption it may release them continuously into the effluent. If the sludge becomes overloaded with organic solutes that compete for virus binding spots, it may provide bursts of free virus with high titers. Thus the overall objectives of the following study have been three-fold: (1) to determine more precisely those elements in sludge solids which remove or inactivate virions; (2) to improve the technology for recovering and assaying adsorbed, infective viruses; and (3) to devise methods for controlling and improving the removal of viruses by sludge solids or other modes related to the recovery of wastewaters. Current interest in the adsorption of virus particles to colloidal and other surfaces remains high. Extensive reviews and discussions have appeared recently on the subject (6-10).

BACKGROUND INFORMATION

Adsorption phenomena have been both bane and boon to detection and monitoring of viruses in waste and other waters. On the one hand, we have the nuisance of viruses which sometimes seem to disappear mysteriously when added to wastewaters and if they do reappear, do so in the wrong dilution tube--results explained by evoking either an adsorption to colloidal materials or clumping and randomized break-up of the virions. On the other hand when we can effect an efficient adsorption of viruses to insoluble carriers of our choice, the phenomenon is used to remove viruses from large volumes

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of water in a nondestructive fashion and to permit quantitation of these low density populations.

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In recent years several sophisticated techniques have been devised which enable us to make more or less quantitative recoveries of viruses from water contaminated with feces. Extensive reviews of this technology have been published at frequent intervals (11-13). Generally these techniques have depended on selective adsorption of the virions to surfaces of filters (14-17), plastic membranes (18-19), colloidal precipitates (20,21) or inorganic particulates such as bentonite or magnetite (22-24). Perhaps the methods in the most advanced stage of development are those which depend on membrane filters to adsorb ether-resistant, water-borne viruses from polluted water. These include the Aquella^R device developed by Wallis and Melnick and their associates (25-28) and the celite-filter combinations devised by Hill et al. (29,30). The use of wound fiber filters or celite makes it possible to concentrate free viruses from extremely large water samples, commonly 50 to 100 gallons, by first removing the membrane clogging solids and then selectively adsorbing the virions. The mechanism of this selective adsorption is poorly understood. Occasionally there are losses of viruses due to irreversible adsorption and our inability to insure that the solids surrender their adsorbed viruses.

Nevertheless this torrential flow past the membranes illustrates the remarkable tenacity with which virions can adhere to surfaces. The shearing forces are relatively immense during the filtration concentration process and the contact angles between the particles and the filter are maintained in the face of a highly variable ionic milieu as well as organic competitors for membrane binding sites (31,32).

Thus by these and other methods microbiologists demonstrate routinely the presence of entero- and other viruses in fecal contaminated water, sewage sludge and treated effluent. Despite this success our knowledge of the actual physical form in which the virions occur in nature remains very slight. In part this is because it is necessary to concentrate the viruses in order to quantitate them. This may destroy the native configuration of the particles themselves or alter the interface between virions and the surface of containers, filters, tubing, etc. Large reactive complexes such as zoogloal floc and clay particles are usually discarded before the viruses are concentrated, perhaps causing the loss of stabilized, infective virions adsorbed to their surfaces. It also seems possible that enteroviruses are transported commonly in fecal pellets which are likely to be excluded by most current water sampling techniques.

The rates of natural die-off of enteroviruses in water and sewage have been determined by many researchers, frequently with a comment aside that either the initial reduction in titer or even the entire loss could be ascribed to adsorption of the virions by colloidal material. Although repeated attempts have been made to correlate both the type and quantity of the total solids content of sewage with the amount of virus lost after seeding, the overall picture of what actually happens to this virus is not clear. In general it is recognized that water with a high fecal organic

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content and an opportunity to fill its biological oxygen demand will reduce or eliminate its population of enteroviruses in a few hours (33-37). On the other hand, a large proportion of the viruses which survive the initial reduction at the treatment plant and reach the outfall, will exhibit half-lives measured in days or weeks instead of a few hours (34,38). There appear to be two types of virus adsorption, reversible and irreversible. Reversible adsorption occurs when viruses are adsorbed to clays (24,39,40), inorganic precipitates (41) and vegetable fiber (42). It occurs very quickly after virus is added to the water (33) and responds to manipulation of pH and ionic strength. It is probably a physical phenomenon (33).

The irreversible adsorption is apparently a biological process with a rather long lag time (35-37). It has a pronounced oxygen demand (35,36) and it is not clearly related to any particular bacterial or algal species which has been studied (33). Its response to pH, solvents, and ionic strength is unpredictable and not very pronounced (35,36). There are no definite indications as to whether this virus loss is due to irreversible adsorption or to some other factor(s). It is striking, however, that these losses correspond very well to the characteristics of the ciliate protozoan populations in waste water. These build up exponentially during floc formation and show seasonal variations which might explain unusual peaks of virus occurrence. Further, they have slower generation times than bacteria and their feeding activity is very sensitive to changes in temperature and oxygen levels.

Coproantibodies are induced by polioviruses and probably other enteroviruses (43). There exists the possibility that these IgG and IgA antibodies help reduce the viral population either by direct neutralization or by agglutinating the virions and opsonizing the clumps for grazing microbes. These antibodies may also coat insoluble fecal components and result in adherence of the virions to fiber, cell debris, etc. At the moment, their role in the ecology of enteric viruses is virtually unknown.

There is a wide variety of solid surfaces which interact with water-borne viruses and cause adsorption losses: (1) dispersed detritus, plant and animal products and inorganic suspended solids in natural water; (2) activated sludge from several modifications of the process and its associated waste water; and (3) fecal solids from humans, domestic and wild animals, fish and other sources. These materials are related as a subject and may respond equally to an improved technology for detection and assay of virus in complex mileaux.

Although primary and secondary sewage treatment "removes" or "inactivates" 90 to 99.99 percent of the detectable viruses in raw sewage, we know very little about the ultimate fate of these virions or the mechanisms which remove them from the active inventory. From general experience with colloids it would appear that any group of protein-coated particles ranging in size between 20 and 100 nm which have a rather high net negative charge should interact readily with the diverse mixture of suspended microbes, soil colloids, fragmented plant and animal debris, shredded domestic products, industrial solids and emulsions, etc., which make up raw sewage and

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digester liquors. The most compelling aspect of the phenomenon is that viruses do survive modern waste treatment and continue to pose a genuine threat. This survival is even more remarkable when one recalls that it occurs in an extraordinarily hostile environment filled with powerful microbial enzymes, sticky polyelectrolytes, ammonia, hydrogen sulfide, nucleophilic oxidants, etc. Recent reviews have been published dealing with the detection, occurrence and removal of viruses during waste treatment (44-47), but so far there seem to be few, if any, broad principles which account for both survival and loss of viruses.

Although microbial exocellular polymers are generally negatively charged, they may acquire enough metal hydroxide to provide a cationic surface. Virus particles in contact with these "shaggy" polymer surfaces may be destabilized to the point the virions adsorb to the capsules of other microbes, glass, plastic plates, etc. In the subsequent sets of bridging reactions, a certain proportion of virions remain unadsorbed, depending on the pH, ionic strength and competing soluble organic compounds. This variable population of free virus becomes more understandable if we imagine some of the virions being coated with one polymer molecule, others with two, three, etc. However, these viruses are still flocculated or adsorbed even though all the anionic groups on the surface are not neutralized. Thus the viruses are adsorbed with varying degrees of avidity by surfaces. They may even become restabilized if the polymers anchoring them to surfaces are digested by microbial enzymes, if the particles are subjected to shear forces, or if the bridging cation patches undergo hydrolysis. Table 1 summarizes the heterogeneous flocculation reactions observed with different ratios of particles to flocculent. It suggests one reason why aerated sludge fails to remove viruses completely when the flocs settle.

TABLE 1. POSSIBLE FLOCCULATION REACTIONS FOR COLLOIDAL PARTICLES DESTABILIZED BY A POLYELECTROLYTE FLOCCULANT. (Summarized from Daniels [114]).

Reaction	Physical Response
1. Initial adsorption: optimum ratio of flocculant to particle	Particle destabilized by adsorption of flocculant in varying quantities
2. Unstable particles of (1) interact by bridging polyelectrolyte	Floc particle formed
3. Initial adsorption: insufficient flocculant	Incomplete flocculation
4. Initial adsorption: excess flocculant	Stable dispersed particle suspension formed
5. Secondary adsorption of flocculent due to net reversal of charge by the population coated with flocculant	Initial destabilized particle population restabilized

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The comminution of the solids during aeration provides shearing forces large enough to remove aggregates of virions and perhaps even single particles at the isoelectric point of virus coat proteins.

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SECTION 2

CONCLUSIONS

1. Representative viruses from all the major unenveloped virus groups adhere to bacterial flocs from activated sludge. The sludge bacteria and yeast can be divided into two groups based on their ability or inability to adhere to polystyrene plates. All adherent bacteria adsorb poliovirus type 1 (PV-1) and other viruses to the surface of colonies and individual cells in the presence of Al^{3+} and Mg^{2+} ; nonadhering bacteria frequently do not adsorb viruses or do so less efficiently.
2. Primary sludge solids and MLSS solids could be fractionated by differential centrifugation and sucrose gradient density centrifugation. When ^{125}I -labeled PV-1 was mixed with the solids before centrifugation most of the label was associated with: (1) fractions containing broken bacterial fragments and single cells or (2) fractions containing large clumps and filaments, paper debris, vegetable fragments, etc. The largest number of counts were associated with fragments 1 μm diameter.
3. Polyhydroxybutyrate granules from Zoogloea ramigera exhibited a strong affinity for PV-1. Large quantities of PHB granules occur as a result of lysis induced by the activated sludge process.
4. Exocellular polymers were extracted from four strains of Z. ramigera and one Klebsiella pneumoniae type 3 and purified. The purified exopolysaccharides reacted very strongly with ^{125}I -PV-1 and either precipitated the virions or neutralized them.
5. Adsorption of viruses by Z. ramigera and its cell exopolysaccharides was mediated by the addition of 0.05M Al^{3+} and by Mg^{2+} . Ca^{2+} can not be substituted. These large ions are involved in a complex interaction of the viruses and the cell surface. The data suggest that the first interaction of the virion and the bacterial surface is electrostatic and results in a destabilization of the bacterial surface. This is sometimes represented as a "reversible sorption" and is sensitive to interference or stripping by EDTA. At the end of 60 to 180 minutes, the virus particles can no longer be stripped with EDTA and/or pH 11 glycine buffer. The reversible sorption with the cells was analogous to the reversible sorption seen with membrane filters. The secondary binding is broken only with 4 percent beef extract, pH 9, suggesting that metal hydroxides are being hydrolyzed and specific superficial "domains" of virus protein coat substructures no longer interdigitate with exopolysaccharides or glycoproteins.

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6. Ciliated protozoans have been demonstrated to acquire viruses as they graze on the sludge floc containing adsorbed virions. The virus particles are internalized and shed over a 12 to 48 hr period. Protozoa only acquire viruses which are adsorbed to bacteria. There is no indication that ingested viruses multiply inside the ciliate cells during this period.

7. Virions are released into the effluent as the floc is grazed by ciliates or is broken up by violent aeration. The viruses, still adsorbed to small pieces of capsular polysaccharide are washed out in the overflow. Thus under bulking conditions more viruses are to be expected in the effluent. A reduction in suspended solids may reduce the effluent viral count but will not eliminate single virions (or small clusters) with attached bits of polysaccharide.

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SECTION 3

RECOMMENDATIONS

1. The study should be expanded to include the adsorption of viruses to species of sludge organisms other than Zoogloea ramigera.

A quantitative screening method has been devised to select organisms from sludge flocs which produce heavy glycocalyx and adhere to polystyrene surfaces. Thus subcultures can be chosen which have a high avidity for viruses. ¹²⁵I-labelled viruses can be adsorbed by standardized layers of such adherent cells--typically species of nonpigmented pseudomonads and Bacillus--and be used to determine the most avid organisms. Also, the non-adherent isolates should be characterized for their avidity for metals and viruses.

2. The kinetics of virus removal need to be studied in the laboratory with activated sludge units which have been heavily seeded with the high avidity bacteria. Variables such as pH, metal ion, temperature, aeration rate, C/N ratio, etc., need to be studied systematically.

3. Purified glycocalyx should be isolated from high- and low-avidity isolates and electrophoretic, biochemical and/or serological comparisons made. Klebsiella pneumoniae is frequently found in sludge solids. The chemical structures of K. pneumonia serotypes are well known and would be useful models.

4. An effort should be made to extract relatively large quantities of glycocalyx from different types of sludge solids (aerobic, anaerobic, dried mixed liquor suspended solids, high avidity seed, etc.) and to determine the feasibility of using it to adsorb viruses, heavy metals, organo-metallic compounds and selected materials from the 52 items on the U.S. EPA hazardous substances list. It may be possible to bind the sticky polymers to beads, chips, plates, etc., and to use them as a means of scrubbing treated effluents free of viruses or other hazards.

5. The importance of ciliates as potential vectors for viruses will be investigated further using both pure cultures of Tetrahymena and wild mixed types isolated by flotation methods from MLSS. The role of metal ions and nonpigmented pseudomonads in this adsorption process requires careful study since there is some evidence to indicate that magnesium and cadmium increase removal of poliovirus 1 by mixed ciliates fed Zoogloea. If the preliminary indications of significant virus removal by ciliates can

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be supported, an attempt should be made to follow the fate of the ciliate-virus combinations up the food chain. It is hoped that an effort will be made to determine virus turnover time for ciliates such as Vorticella. Autoradiographic methods can be used to see if the double-labeled virus on ciliates or Zoogloea are transferred to higher forms such as nematodes, rotifers and fish.

6. Human polyoma tumor viruses are probably shed in vast numbers in urine and excrement. It is unclear how 85 percent of the population can show seroconversion with the virus being cryptic in all cases. It is possible that it is inactivated by adsorption to gut bacteria. A reasonably broad screening of potential sources of human polyoma viruses should be conducted using raw sewage, pH 10 eluates from sludge solids, treatment plant effluent, combined storm overflow, fresh stools from normal and immunosuppressed patients and urine from general hospital laboratories.

A limited number of references suggest that SV₄₀ virus is not inactivated in sewage or during primary and secondary treatment. However, the data are 10 years old and it is necessary to determine survival of BK virus, RF virus, mouse polyoma and Rat K viruses under more controlled conditions of treatment. A special effort will be made to determine the efficiency of recovery techniques for the viruses which have been seeded into sewage and activated sludge. Total recovery of ¹²⁵I-labeled papoviruses vs. PFU could be determined over 24 to 72 hour periods. The effects of elevated temperature and anaerobic fermentation on their survival need to be studied.

7. The fate of 0.1 to 0.5 μ m PHB granules in activated sludge treatment needs to be described. It is unclear whether they are digested during sewage treatment or if they end up in the effluent as virus carriers.

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SECTION 4

MATERIALS AND METHODS

ORGANISMS

The following animal viruses (Table 2) and pure cultures of Zoogloea ramigera (Table 3) were used:

TABLE 2. VIRUSES

Virus	Cell Culture Line	Reference
Adenovirus 7	HEK	(48)
Adenovirus 31	HEK	(48)
Coxsackie B3	BGM	(49)
Echovirus 11	HEK	(48)
Poliovirus 1 (Sabin K-1)	BGM, Vero	(49)
Poliovirus 1 (Mahoney)	BGM, HEp2	(49)
Reovirus 1	BGM	(49)
Coliphage f2	<u>E. coli</u> A19*	

* Escherichia coli A19 = RNase mutant

TABLE 3. ZOOGLOEA RAMIGERA

Strain	Amer. Type Culture Collection Number	Reference
I-16-M	ATCC 19623	K.T. Crabtree (50)
106	ATCC 19544	N.C. Dondero (50)
115	ATCC 25935	B.A. Friedman, P.R. Dugan (50)
216	ATCC 19123	N.C. Dondero (50)

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The Zoogloea cultures were maintained at room temperatures in stab butts of BBL trypticase soy agar plus 0.5 percent Difco yeast extract and 2.0 mg/mL vitamin B₁₂ and transferred at intervals of three months.

Three monoprotozoan cultures were obtained (Wards Natural Sciences): Paramecium aurelia and P. caudatum, which were both monoxenic with a putative Aerobacter aerogenes, and Colpidium sp. which was axenic. Colpidium and P. caudatum grew slowly only on Z. ramigera grown in proteose peptone-yeast extract but not in Bacto tryptic soy glucose broth. The zoogloal cells were washed with PBS and inoculated directly (2×10^6 cells/mL) into the Colpidium growth medium: Brewer's yeast powder, 10 g; glucose, 5 g; proteose peptone, 20 g; pH 7.0. The washed cells were fed to the paramacia in cerophyll medium: cerophyll powder, 1.5 g boiled 5-10 minutes and sterilized by filtration through a Millipore HA filter, 0.45 nm pore size; addition of a "nondialyzable yeast extract" (51) reduced the need for constant replenishment of the zoogloea.

GENERAL PROCEDURES

Tissue cultures

Vero and BGM cells were grown in a 1:1 mixture of MEM medium and L-15 medium (Leibovitz) using 10 percent fetal calf serum and 1 percent of a 7.5 percent NaHCO₃ stock (49). Human HEP-2 cells were grown in MEM above plus 5 percent fetal calf serum. Human fetal embryonic kidney (HEK) cells were obtained from Flow Laboratories and grown for 3 to 5 passages in MEM plus 10 percent fetal calf serum and stored at -192°C. Addition of gentamicin (5 µg/mL) to the growth media for BGM cells markedly improved the growth and plaquing capabilities of these cell lines.

Plaque formation

E. coli A19 was grown for 17 hr at 37°C with aeration in trypticase yeast extract medium (TYE) (50): 10 g Bacto tryptone, 8 g NaCl, 1 g yeast extract, 1 g glucose and 0.22 g CaCl₂/L. Base layers of TYE contained 1.5 percent agar; 2.5 ml of TYE agar (0.7 percent agar) was used for the overlay and seeded with 0.01 mL E. coli A19. The plates were inverted and incubated for 6 hrs at 37°C. A19 cells can be stored at -65°C ready for use, rather than being prepared each day (52).

Vero, BGM and HEP-2 cells were used for plaque assays as described by Dahling et al. (49) using an agar overlay containing MEM L-glutamine, MEM nonessential amino acids, and 2 percent fetal calf serum; each 100 mL contained 3.0 mL 7.5 percent NaHCO₃, 1.0 mL 1 percent MgCl₂ H₂O solution, 1.5 mL 0.1 percent neutral red solution, 200 units penicillin, 100 µg streptomycin and 50 µg mycostatin. HEK cells were overlaid with MEM, 5 percent fetal calf serum, pH 7.4. All agar overlays contained either 0.8 percent ion agar No. 2 (Oxoid) or 0.8-1.0 percent Noble agar (Bacto). Reovirus plaque formation was enhanced by incorporation of 0.016 percent pancreatin, into the overlay medium (55). Plaque formation was observed in 60 mm polystyrene plates and in Multiwell (Falcon) plates (53, 54).

Microtiter TCID₅₀ endpoint assays and CPE

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Techniques described by Conrath (56) were used for viral neutralization and metabolic inhibition tests of virus concentrates eluted from sludge solids. Microtest II plates were employed and the cell monolayers were stained with Giemsa reagent according to the following schedule (57): after 2 to 10 days the Microtest II culture plates were washed by immersion in PBS and fixed by immersion in methanol 5 minutes. The monolayers were washed 15 minutes in dilute Giemsa solution (stock Giemsa diluted 1:15) and rinsed 20 to 40 seconds in tap water and distilled water. The plates were inverted and air-dried. (Stock Giemsa: 1 g Giemsa powder was dissolved in 66 mL glycerol at 55°C for 1 hr., and 66 mL methanol was added.)

Methods of identification

A two-step scheme was employed to make quantitative estimates of the number of viruses in each of five groups: polio-, coxsackie B-, echo-, adeno- and reoviruses. A presumptive test of the virus concentrates was made first in Microtest II plates to determine which groups were represented in the sample and what was their approximate TCID₅₀ titer. The dilution sequences, the challenge with antipoliiovirus IgG (rabbit) and the cell sensitivity spectrum are illustrated in Figure 1. The interpretation of the patterns obtained by this procedure is shown in Figure 2. Generally it was possible to differentiate the virus groups even when they occurred in mixtures by suppressing one or two groups with antiserum or a nonpermissive host. In those samples where a fourth or unrecognized component was present the samples were plated and the individual plaques were picked with sterile tooth picks and a confirmed test was applied. The unknown plaques were subcultured in microtiter wells, the resulting virus was passed back through the screen (Figure 1) and/or the cytopathology of the infected cells was examined by staining with MGG stains. If adeno- or reoviruses were suspected, the plaques were grown in 4-chamber Lab-Tek well slides and stained with acridine orange as well as MGG.

Sludge solids sources

(1) Meadowbrook-Limestone Treatment Facility is an activated sludge process with a peak flow rated at 12 MGD. The activated sludge aeration tanks maintain 2 ppm dissolved oxygen, provide six hours detention time at average flow, including 30 percent activated sludge recirculation. Aerobic digesters provide twenty days detention of sludge with complete mixing of tanks contents. Decanting tanks are provided to thicken digested sludge prior to pumping to drying beds. (2) Metropolitan Syracuse Treatment Plant is a primary settling and contact stabilization process with polyelectrolyte precipitation. The plant's average flow is 62 MGD. Precipitated solids are pumped to primary and secondary anaerobic digesters which have detention times of 14.5 and 4.4 days, respectively.

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Samples were collected at various periods to determine if seasonal fluctuations in virus content occurred with the onset of cold weather.

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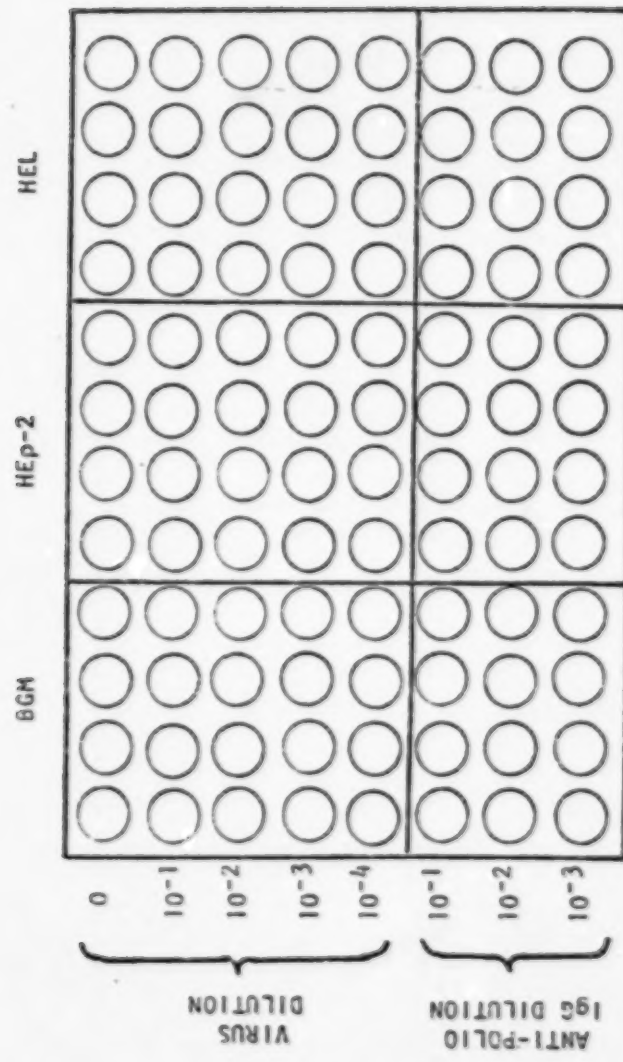
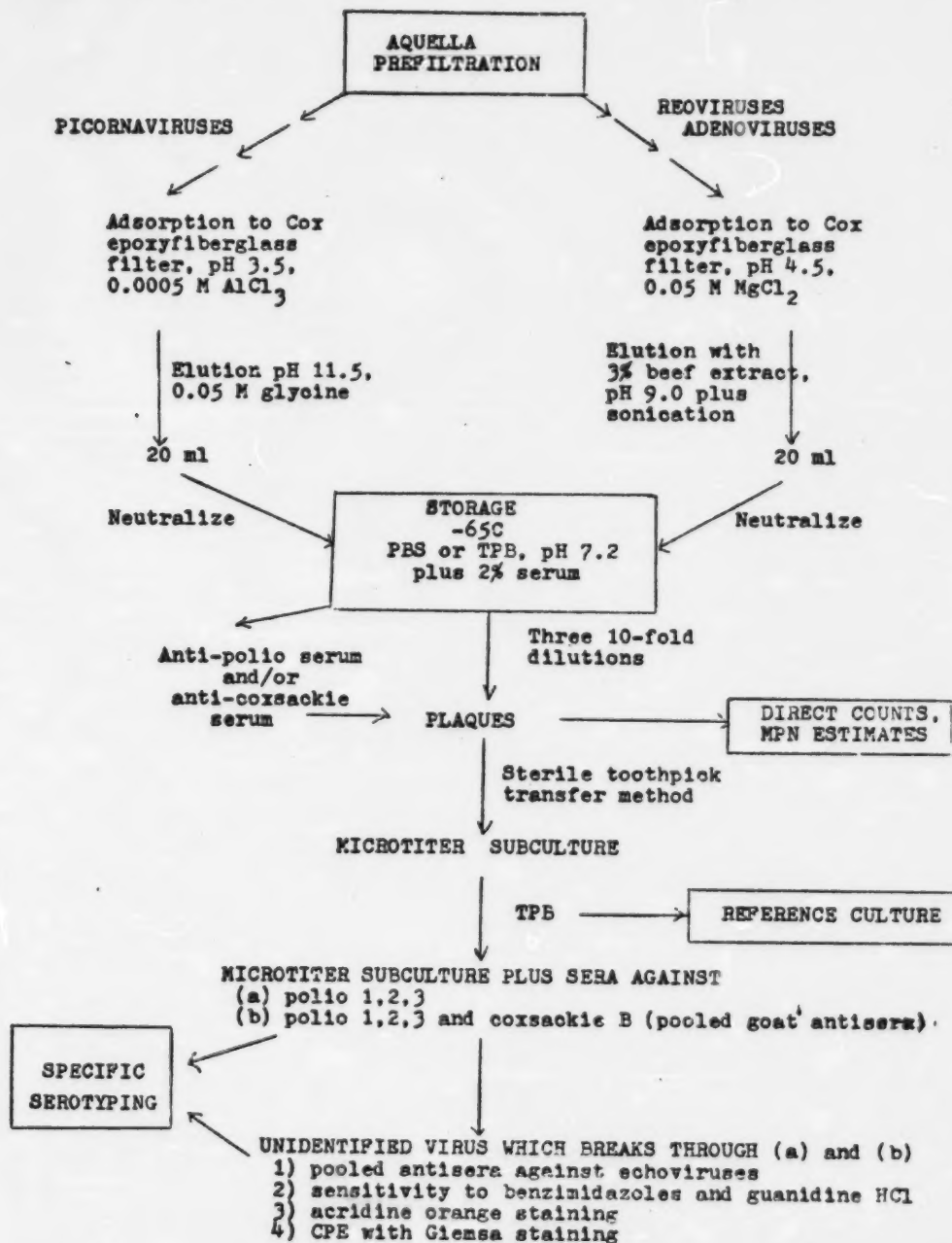


Figure 1. Presumptive assay for viruses from activated sludge samples.. (Micro Test II plate).

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Figure 2. Identification and quantification of viruses in storm waters after concentrating them with the Aquella instrument. Cell culture: HEp-2 and BGM cells.



Grab samples were collected and stored at 4°C for 17 hr or -20°C. Aliquots of concentrated sludge solids were frozen at -65°C and used in different experiments to avoid daily fluctuations in floc quality. Raw sludge samples and digested samples were collected within a few minutes of each other. Unless otherwise specified the samples were collected between 9:30 and 11:00 AM.

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FRACTIONATION OF SLUDGE SOLIDS

The solids in undigested sludge, digested sludge and mixed liquor were size-fractionated by the continuous centrifugation procedures of Rickert and Hunter (58,59) into four fractions (Table 4).

TABLE 4. FRACTIONATION OF SLUDGE SOLIDS BY DIFFERENTIAL CENTRIFUGATION.

Fraction	Ideal size limits	Collection
Settleable	100 μ m	1 x g sediment*
Supra-colloidal	1-100 μ m	4,800 x g pellet*
Colloidal	1 nm-1 μ m	62,500 x g pellet*
Soluble	1 nm	62,500 x g supernatant*

* flow rate 100 ml/min.⁻¹

The individual fractions were refined further by subjecting them to sucrose density gradient centrifugation in 14 to 65 per cent sucrose gradients containing 0.01 M sodium phosphate buffer, pH 7.2. The samples were centrifuged 90 minutes at 22,000 RPM in a Spinco No. 30 rotor, 4°C, or a SW39 rotor. Liquid fractions were removed from the gradients by insertion of a needle to predetermined levels in the tubes; viscous or clogging particulate fractions were collected by flash freezing the tubes, removing the frozen core and slicing it into sections with a taut, heated pianowire knife. Sucrose was removed from the fractions by microdialysis or by pelleting the particulates at 120,000 x g, decanting and resuspending the solids in PBS.

Labeling of poliovirus 1 with ¹²⁵I

Poliovirus was grown in BGM cells which were removed by shaking when the infected cells rounded up (36 hrs). The infected cells were centrifuged at low speed, lysed by three cycles of freezing and thawing. The virus was collected on a 65 percent sucrose bumper after centrifugation 1 hr at 105,000 x g in a discontinuous gradient: 65 percent sucrose, 1 part; 25 percent sucrose, 3 parts. The virus at the interface was re-purified by centrifugation to equilibrium on 14 to 65 percent preformed

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gradients of sucrose--0.01 M phosphate buffer (pH 7.2). Maximum adsorption at 280 nm was consistently obtained in the 25 percent sucrose fractions. This fraction was pelleted at 105,000 x g for 1 hr, in a Spinco No. 40 rotor and allowed to elute spontaneously in PBS, pH 7.4 overnight.

The poliovirus was labelled by either of two techniques: (1) the lactoperoxidase-catalyzed iodination of virion surface proteins or (2) the chloroglycouril (Iodogen[®]) method for membrane iodination. The first method, described by Li and Fox (60), provided heavy labeling with ^{125}I without inactivating the virions. One-half ml of the virus sample above (5×10^{10} PFU/ml) was mixed with 5 μM H_2O_2 and addition of 5 μM H_2O_2 at one minute intervals for 5 minutes. The reaction was performed in a water bath at 30°C with agitation and terminated at 4°C by addition of 1 mM mercaptoethanol. The labeled virus was pelleted at 105,000 x g and resuspended in PBS, pH 7.2.

In the second labelling method the externally exposed proteins of the viral capsid were iodinated with 1,3,4,6-tetrachloro-3,6-diphenylglycouril (chloroglycouril or Iodo-gen, Pierce Chemical Co., Rockford, IL) (61,62) under conditions that label both milligram and microgram quantities of proteins (63) with about 3×10^5 dpm of ^{125}I per μg of protein (approximately 3×10^{10} PFU). One hundred aliquots of virus (200 μg viral protein in 10 mM Na_2HPO_4 , pH 7.4) were reacted with 500 μCi of carrier-free Na^{125}I at 0°C, 10 min, in 13 x 75 mm tubes coated with 10 μg chloroglycouril (61). The reaction was stopped by removal of the virus from the reaction vessel and rinsing with two 100 μL aliquots of 10 mM Na_2HPO_4 , pH 7.4.

Recovery of viruses from sludge solids

Sludge solids were concentrated (1) on the surface of cellulose acetate 10 nm "Fulflo" depth filters (Commercial Filters, Carborundum Co., Lebanon, IN) treated with a 0.1 percent Tween 80 for 18 hours or (2) by settling overnight at 4°C, decanting the supernatant and centrifuging the settleable solids at 6,000 RPM in the Sorvall SS-1. Two techniques were used to remove polioviruses from the sludge solids and to concentrate them.

(1) Freeze-thawing. The solids were resuspended in equal volumes of TPB or 2X Eagles MEM with Earles basal salts, pH 7.2 and subjected to four cycles of freezing and thawing. The resuspended solids were distributed in approximately 50-ml lots in centrifuge tubes and frozen by immersion in an ethanol-dry ice bath. The frozen samples were thawed at 37°C and centrifuged. The supernatant was collected after each freezing and the solids were resuspended. The combined virus supernatants were adjusted to pH 7.2 with 0.12 M HCl, sterilized with chloroform for six hours, aerated and centrifuged. After the addition of 2 percent fetal calf serum and 1 percent MgCl_2 , the samples were stored at -40°C.

(2) Elution at pH 11.5 (27, 64). Packed sludge solids were resuspended in 0.05 M glycine buffer, pH 11.5 at a ratio of 1 g wet solid: 5 ml

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buffer. The solids were blended for 3 minutes. Small samples were clarified by 5 min. centrifugation and large ones by rapid filtration through 1 μ m orlon clarifying filters or through Millipore A25 prefilter pads. Aluminum chloride was added to the supernatants and filtrates to 0.003 M and the pH was adjusted to 5.0 with N/1 HCl and stirring. The resulting aluminum floc was allowed to settle 30 minutes and was decanted and pelleted by centrifugation at 4,000 RPM, 10 minutes. The floc was solubilized with 3-5 mL of 0.005 M EDTA in pH 3.5 glycine, 0.05 M, with phenol red indicator. Radioactive samples were assayed directly in the sparkling suspension. The aluminum floc which contained EDTA and toxic organic materials was not compatible with tissue cultures. Consequently, those samples which were to be assayed for PFU were layered on top of a two-layer sucrose gradient consisting of 25 percent sucrose and a 55 percent sucrose bumper in 0.12 M isotonic saline. These samples were centrifuged at 22,000 x g for 90 to 180 minutes and the virus at the interface was collected, diluted and assayed. Samples containing sucrose (diluted with HMEM) could adsorb to monolayers and the adsorption media containing sucrose was decanted before the agar overlayment was made.

Virus concentration

Five gallon sludge or sewage samples were decanted and viruses in the supernatant were isolated by selective adsorption to membrane filters. The Aquella Virus Concentrator (Carborundum Co., Niagara Falls, N.Y.) was used to remove the remaining solids and to adsorb the viruses to a "sandwich" of three cellulose acetate membrane filters--5 μ m, 2 μ m and 0.45 μ m porosities. Orlon (10 and 1 μ m porosities) and cellulose acetate (0.8 μ m) wood textile filters "Fulflo" were used "in series" ahead of the membrane filters (65). Virus samples were adjusted to pH 4.5 with HCl and $MgCl_2$ (0.05 M) was added after the prefiltration step. The adsorbed viruses were eluted from the membrane with 0.05 M glycine, pH 11.5 or 3 percent beef extract, pH 9.5. Eluates were collected in cold buffer, pH 7.2 to prevent damage to the virions (individual virus particles) and sterilized with chloroform. Two percent fetal calf serum and one percent $MgCl_2$ were added to the preparations which were then frozen and stored at $-40^{\circ}C$.

Production of *Z. ramigera* exocellular polymers

Production was by growth in Bacto trypticase soy broth or by the general method of Crabtree (66): the Zoogloea were cultured 7 to 14 days in an arginine medium (arginine-HCl, 0.5 g; alanine, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; K_2HPO_4 , 2.0 g; KH_2PO_4 , 1.0 g; glucose, 20 g; vitamin B_{12} , $1.5 \mu g$) at $22^{\circ}C$ with shaking on a reciprocal shaker. Only cultures showing strong microbial flocculation were used to produce the polymer.

The methods devised by P.R. Dugan and his associates (67-69) were employed with minor modifications for purification of the exocellular polymer. One hundred ml of 7 to 14 day *Z. ramigera* culture was mixed with 3 volumes acidified EtOH, $4^{\circ}C$. When chilled overnight the polymer floated to the surface and could be removed by skimming with a watch glass. Additional polymer was removed by centrifugation at 1300 x g, 1 hr. The polymer was

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dissolved in one-third the original volume of cold distilled H₂O for 17 hrs and then centrifuged at 1300 x g 10 minutes. The acid alcohol wash and cold water precipitation was repeated. The sample was washed with 0.01 M EDTA, pH 6.5 and dialyzed against several changes of double distilled water, 4°C, for 48 to 72 hrs, and stored at 4°C with sodium azide. Dried polymer was prepared by reprecipitation with two volumes of acetone at -15°C, collected by centrifugation, washed with three changes of -15°C acetone and air-dried.

Polyhydroxybutyrate granules

Zoogloea ramigera was grown with shaking for 48 hr, 30°C, in a defined basal medium to promote dispersed growth: arginine 0.5 g; MgSO₄ 7H₂O 0.2 g; K₂HPO₄ 2 g; KH₂PO₄ 1 g; vitamin B₁₂ 2 ng/mL; biotin 2 ng/mL. Addition of 5 g glucose/L and further incubation for 10 hr caused the cells to floc and resulted in maximum accumulation of PHB (69). Between 40 and 50 percent of the dry weight of the cells was PHB.

Whole, undegraded PHB granules were isolated from the cells by treatment with lysozyme (70,71). The cells were pelleted at low speed, washed with PBS, pH 7.0 and resuspended in 30 ml PBS containing 33 mg lysozyme (Mann), 0.5 ml 1M MgCl₂ and 0.35 mg DNase (Sigma), 30 min. The lysate was sonicated (20-kc Bronwill Biosonik probe) 2 min and the PHB granules were isolated by overlaying the lysate on glycerol, centrifuging 20 minutes at 9,000 x g and collecting the PHB granules at the interface. The granules were resuspended in 0.05 M tris-HCl, pH 8.0 and the process was repeated twice. The preparations were dialyzed 16 hrs against PBS, pH 7.4 to remove glycerol and stored at 4°C.

Gamma counting

¹²⁵Iodine was measured in a Nuclear-Chicago automatic gamma well counting system consisting of a 1085 controller and a 8725 analyzer unit. All samples were automatically corrected for background radiation.

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SECTION 5

EXPERIMENTAL RESULTS

ADSORPTION AND RECOVERY OF VIRUSES FROM TREATMENT PLANT SLUDGES

Recovery of wild viruses from treatment plant sludges

Tables 5A, 5B and 5C summarize the titers of chloroform-resistant viruses which are typical for the sewage and sludges used in this study. More than half the samples had significant levels of viruses. A total of 697 individual virus plaques were isolated and identified. It is apparent that most of the PFU of virus were either poliovirus or other enteroviruses. Although techniques were employed which can recover adenoviruses rather efficiently--ie., adsorption at pH 4.5 and elution at pH 9.5--only a small number of adenovirus isolates were observed. The use of HEp-2 cells and HEL cells, which are not very efficient for some strains of adenovirus, may have contributed to the infrequent isolations.

The high frequency of poliovirus isolations almost certainly represented Sabin vaccine virus for the most part. Thirty-three random isolates were unable to form plaques at 40°C, a test which is usually indicative of Sabin poliovaccine (72). The other enteroviruses were identified and both coxsackie B and echoviruses were isolated (on the basis of their plaque morphology and host cell predilection). Reoviruses are frequently indicative of animal waste such as feed-lot runoff and slaughter house waste. There was very little input from these sources into the treatment plants under study. Further, quantitative isolation of these particular viruses is still in the developmental stage and there are few data in the literature to serve as guidelines.

The levels of viruses found in the sludge samples were comparatively high in some samples from the Syracuse Metropolitan Treatment Plant, particularly samples 1.0-5A and 1.0-5C. Comparable types of recoveries in the literature are about the same order of magnitude: Dahling et al. (49) reported that raw sewage from many localities rarely contained more than 100 PFU/100 mL; Metcalf et al. (73) found effluents from two activated sludge treatment plants contained 3 to 23 PFU/gal over several months; Cliver (74) reported 1 to 17 PFU/100 ml in raw sewage and 0 to more than 400 PFU/100 mL in digested sludge samples.

Raw primary sludge supernatant from the Syracuse Metropolitan Treatment Plant had high values of 90 PFU/100 mL and an average content of 25

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PFU/100 mL; Meadowbrook-Limestone samples had a high value of 52 PFU/100 mL and an average of 15 PFU/100 mL; Ley Creek sample data are inconclusive but appear to be about 15 to 100 PFU/100 mL. The digested samples had comparable values but showed a tendency to be slightly lower than raw sludge. The data did not suggest strong viricidal effects as a result of sludge digestion. The data in Tables 5A-5C indicate that the solids fractions have about the same virus content as the free liquid phase of either raw or digested sludge. Since the solids pellets represented only 8 to 20 percent of the volume of the sludge these superficial data provide some confidence in the belief that the solids are strong natural concentrators.

A seasonal trend was observed with higher counts in the fall samples. This may reflect the compulsory immunization of school children with Salk vaccine prior to their admission to school. Immunized children continue to shed live polioviruses into the GI tract for five or six weeks after inoculation.

Grab samples such as these provide irregular titers of virus since the transmission of GI tract viruses through the sewer system results in irregular bursts, depending on the number of shedders, the dilution factor, flow mixing, temperature, viricidal wastes, etc. Occasional samples with very high titers occur because viruses may form concentrated foci when they are inside exfoliated cells which were shed into the GI tract, adsorbed to vegetable fibers or trapped by a colloidal matrix. Thus we see that pairs of samples such as 2.0-5B which were collected within minutes of each other from the same tap show a five-fold difference in titer. Samples collected 2 hours apart (5.1-5A and 5.2-5A; 5.1-5B and 5.2-5B) may or may not show such large variations.

The failure to remove viruses by adsorption does not seem due to lack of binding sites entirely. Table 6 shows that activated sludge solids from the same experiments described in Table 5A were capable of adsorbing virus under laboratory conditions. Aliquots of a frozen stock of poliovirus 1 were added to 500 mL of undigested sludge which was aerated 48 hours and sampled at 4 to 8 hour intervals for free poliovirus. After an initial change in titer within the first 5 minutes, an exponential decrease in titer was observed. Something of a negative correlation appears in this experiment between the slope and the free virus in the supernatants of 2A. Those samples with a low free virus content at the Metropolitan plant (Table 5A) had the highest binding constants in the laboratory. Conversely, those sludge samples with the highest free virus content had the lowest binding constants.

Fractionation of raw sludge

Raw, undigested sludge was seeded with ¹²⁵I-labelled poliovirus 1, aerated for 19 hrs and then fractionated first by differential centrifugation and then by sucrose density gradient centrifugations of the previous fractions (Table 7). The virus label was heavily associated with the supracolloidal fraction (1-100 μ m) and to a lesser extent with the settleable fraction. The viable recovery data showed that very little label in

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TABLE 5A. VIRUS ISOLATES FROM METROPOLITAN TREATMENT PLANT. ***

Sample Date	Virus	Supernatant		Solids Associated	
		Raw Primary Sludge 11-1-100	Digested Primary Sludge 11-2-100	Raw Primary Sludge 11-1-100	Digested Primary Sludge 11-2-100
1.0-5A 8/18	Total PFU	90	148		
	Poliovirus	20	120		
	Enterovirus*	63	47	ND**	ND**
	Adenovirus	0	4		
	Reovirus	0	0		
	Other	0	0		
2.0-5A 9/12	Total PFU	0	0	0	0
	Poliovirus	0	0	0	0
	Enterovirus*	0	0	0	0
	Adenovirus	0	0	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0
3.0-5A 10/21	Total PFU	22	2000	34	2000
	Poliovirus	15	-	13	-
	Enterovirus*	8	-	20	-
	Adenovirus	0	2	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0
4.0-5A 12/8	Total PFU	19	3		12
	Poliovirus	8	0		1
	Enterovirus*	1	3	ND**	0
	Adenovirus	8	0		0
	Reovirus	0	0		0
	Other	2	0		11
5.1-5A 12/15 12 AM	Total PFU	13	4	7	1
	Poliovirus	3	0	1	0
	Enterovirus*	10	4	6	1
	Adenovirus	0	0	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0
5.2-5A 12/15 2 PM	Total PFU	8	3	2	0
	Poliovirus	0	0	0	0
	Enterovirus*	4	0	2	0
	Adenovirus	0	0	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0

*Enterovirus = total enterovirus PFU minus poliovirus PFU.

**ND = not done

***Data are plaque forming units (PFU)/100 mL of raw primary sludge or digested primary sludge.

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TABLE 5B. VIRUS ISOLATES FROM MEADOWBROOK-LIMESTONE CREEK TREATMENT PLANT.***

Sample Date	Virus	Supernatant		Solids Associated	
		Raw Secondary Sludge 10-1-030	Digested Secondary Sludge 10-3-03C	Raw Secondary Sludge 10-1-030	Digested Secondary Sludge 10-3-030
1.0-5B 8/18	Total PFU	31	3		
	Poliovirus	9	0		
	Enterovirus*	20	0	ND**	ND**
	Adenovirus	4	0		
	Reovirus	0	0		
	Other	0	0		
2.0 5B /12	Total PFU	52 10		69	
	Poliovirus	36 8		40	
	Enterovirus*	15 0.5	Not Supplied		Not Supplied
	Adenovirus	0 0			
	Reovirus	0 0		0	
	Other	0 0		0	
3.0-5B 10/21	Total PFU	0	0	0	14
	Poliovirus	0	0	0	0
	Enterovirus*	0	0	0	0
	Adenovirus	0	0	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0
4.0-5B 12/8	Total PFU	0	0	0	0
	Poliovirus	0	0	0	0
	Enterovirus*	0	0	0	0
	Adenovirus	0	0	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0
5.1-5B 12/15 12 AM	Total PFU	3	5	0	4
	Poliovirus	3	0	0	0
	Enterovirus*	0	0	0	4
	Adenovirus	0	5	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0
5.2-5B 12/15 2 PM	Total PFU	8	0	0	0
	Poliovirus	0	0	0	0
	Enterovirus*	8	0	0	0
	Adenovirus	0	0	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0

*Enterovirus = total enterovirus PFU minus poliovirus PFU.

**ND = not done

***Data are plaque forming units (PFU)/100 mL of raw secondary sludge or digested secondary sludge.

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TABLE 5C. VIRUS ISOLATES FROM LEY CREEK TREATMENT PLANT.***

Sample Date	Virus	Supernatant		Solids Associated	
		Raw Combined Sludge	Digested Combined Sludge	Raw Combined Sludge	Digested Combined Sludge
1.0-5C 8/18	Total PFU	100			
	Poliovirus	Toxic	Toxic		
	Enterovirus*	Toxic	Toxic	ND**	ND**
	Adenovirus	Toxic	Toxic		
	Reovirus	0	0		
2.0-5C 9/12	Total PFU	0	21	1	96
	Poliovirus	0	1	0	ND**
	Enterovirus*	0	20	0	0
	Adenovirus	Toxic	Toxic	0	0
	Reovirus	0(toxic)	0(toxic)	0	0
	Other	0	0	0	0
3.0-5C 10/21	Total PFU	5	15	0	3
	Poliovirus	1	0	0	0
	Enterovirus*	4	15	0	0
	Adenovirus	0	0	0	0
	Reovirus	0	0	0	0
	Other	0	0	0	0

*Enterovirus = total enterovirus PFU minus poliovirus PFU.

**ND = not done

***Data are plaque forming units (PFU)/100 mL of raw combined sludge and digested combined sludge.

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TABLE 6. REMOVAL RATE OF POLIO-
VIRUS 1 WITH ACTIVATED SLUDGE
SOLIDS (METROPOLITAN TREATMENT
PLANT). *

Sample	k
1.0-5A	1.3×10^{-3} PFU/min
2.0-5A	3.7×10^{-2} PFU/min
3.0-5A	3.8×10^{-4} PFU/min
4.0-5A	3.6×10^{-3} PFU/min
5.1-5A	2.9×10^{-2} PFU/min
5.2-5A	2.8×10^{-2} PFU/min

* MLSS were adjusted to 2500
mg/l with aerated tap water
and inoculated with 5.8×10^6
PFU/ml. (Residual chlorine
content unknown.)

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TABLE 7. ASSOCIATION OF ^{125}I -LABELED POLIOVIRUS 1 WITH VARIOUS FRACTIONS FROM RAW, UNDIGESTED SLUDGE.

Fraction from Differential Centrifugation	Sucrose Gradient		DPM ^{125}I Labeled Poliovirus 1	Percent of Total Label Adsorbed	PFU $\times 10^5$
	Fraction	% Sucrose			
1. Soluble (Resists pelletting at 62,500 $\times g$)	1A	65	0		.001
	1B	43	833		.001
	1C	25	1,610		.001
	1D	22	703		.001
	1E	17	51		.001
Subtotal			3,197	7.52	
2. Colloidal (Pellets at 62,500 $\times g$)	2A	65	609		.001
	2B	43	824		.001
	2C	25	1,184		20
	2D	22	1,011		80
	2E	17	10		.001
Subtotal			3,638	8.56	
3. Supra- colloidal (Pellets at 4,000 $\times g$)	3A	65	8,039		89
	3B	43	3,334		46
	3C	25	9,852		650
	3D	22	6,719		730
	3E	17	8,528		123
Subtotal			28,433	66.89	
4. Settleable (Pellets at 1 $\times g$)	4A	65	3,479		26
	4B	43	2,010		184
	4C	25	3,813		70
	4D	22	1,002		33
	4E	17	1,052		493
Subtotal			7,248	17.03	
Total			42,508	100.00	

* The sludge was seeded with 8×10^9 PFU/mL, aerated 19 hrs, 30°C , and fractionated by differential centrifugation. Pellets from the fractions were resuspended in 0.01M phosphate buffer, pH 7.0, overlaid on 11 to 65 percent sucrose gradients, and centrifuged 90 min at 22,000 RPM in Spinco rotor #30.

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the fractions was free virus. The infective titers of the heavily labeled C fractions remained very low—less than 4 percent of total PFU applied—suggesting the free virions had been inactivated in some fashion. Several questions are raised by the observations which cannot be answered now: what is the meaning of the even distribution of counts throughout the sucrose in 3A-3E? Is the syrupy polymer material from fraction 4 actually distributed in a reproducible fraction throughout the sucrose gradient? What is the reproducibility of the experiment? Where is the bulk of the bacteria and protozoa in these fractions? The answers to these and related questions became apparent in later experiments.

Sucrose gradient fractionation of sewage treatment solids

Because of limited resources for sampling and biochemical assays it was desirable to use aliquots of a small number of defined sewage samples repeatedly. Solids from the Metro primary aeration tank, the secondary clarifier and the anaerobic digester were studied for their ability to adsorb poliovirus before freezing and after storage at -20°C for 35 days (8/19) or 21 days (10/13) (Table 8). The same batch of labeled virus was used in all cases. No significant differences were noted in primary adsorption (5 hrs) by either fresh or frozen samples.

Centrifugation profiles of ^{125}I -poliovirus seeded into anaerobic sludge samples showed that the virus was largely concentrated in the supracolloidal fraction of the Rickert-Hunter classification (Table 9). From 54 to 63 percent of the label was in fraction 3 of all 4 samples. This fraction contains individual bacterial cells and protozoa. Fraction 2, which would normally include free viruses, had an unexpectedly high ^{125}I count—10 to 24 percent. Although this fraction contains some inorganic colloids, its principle identifiable constituents are microsomal fragments, fractured cell walls, and polymeric filaments. One other source of the Fraction 2 counts must be considered besides the virions. The iodination of poliovirus distributes about three-fourth of the counts on VP-1 proteins of the virus coat and one-fourth on the lactoperoxidase enzyme as a result of self-iodination (61). The poliovirus used in these preparations undoubtedly was contaminated with appreciable quantities of ^{125}I lactoperoxidase and the fraction 2 material may have these origins. However, it seemed more likely that fraction 2 counts may represent partially digested polymer flocs protozoan excreta or "P" substance. In later experiments the poliovirus was labelled by the "Biogen" technique which eliminated the self-iodination problem.

Attempts were made to fractionate sewage sludge by means of sucrose gradients in an effort to trace the principal repository for adsorbed poliovirus. Table 10 shows that surprisingly good reproducibility was achieved for replicates originating from either the resuspended 1 x g pellet or the resuspended 4,800 x g pellet. Since 30 percent of the supracolloidal fraction is at the top of the gradient tube (fraction 3A), there is reason to believe that some self-iodination of the lactoperoxidase occurred. When resuspended 30 percent of the supracolloidal fraction settled to the 65 percent bumper, suggesting that this fraction aggregates

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TABLE 6. ABILITY OF FRESH AND FROZEN SEWAGE SOLIDS TO ADSORB POLIOVIRUS 1.^a

Solids	Experiment	Solids mg/L	Treatment	Adsorption pH 6.8		Elution pH 11.5	
				Influent PPU/L	Unadsorbed PPU/L	Elution PPU/L	Percent Recovery
MLSS (Aeration tank)	6/19	1,620	fresh frozen ^a	2.6x10 ⁶	1.9x10 ³ 2.2x10 ³	1.7x10 ⁶ 1.4x10 ⁶	65 53
	10/13	1,650	fresh ^b frozen ^c	2.6x10 ⁵	1.8x10 ⁴ 1.7x10 ⁴	1.2x10 ⁶ 1.7x10 ⁶	46 65
Raw Sludge (Secondary clarifier) ^c	8/19	22,300	fresh ^a frozen ^a	2.6x10 ⁵	5.6x10 ³ 8.2x10 ³	1.6x10 ⁶ 1.5x10 ⁶	62 58
	10.13	26,800	fresh ^b frozen ^b	2.6x10 ⁵	9.0x10 ³ 7.2x10 ³	7.8x10 ⁵ 1.4x10 ⁶	30 54
Digested Sludge (Anaerobic digester)	8/19	70,700	fresh ^a frozen ^a	2.6x10 ⁵	1.0x10 ² 1.2x10 ³	6.0x10 ⁵ 1.3x10 ⁶	23 50
	10/13	54,800	fresh ^b frozen ^b	2.6x10 ⁵	2.7x10 ³ 7.5x10 ²	1.3x10 ⁶ 8.2x10 ⁵	50 31

^a stored 35 days, -20°C

^b stored 21 days, -20°C

^c Cheadowbrook-Limestone Treatment Facility

^d Metropolitan Syracuse Treatment Plant

^e Virus was adsorbed 5 hours before elution at pH 11.5.

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TABLE 9. COMPARISON OF VIRUS-CONTAINING SUCROSE GRADIENT
PROFILES OF FOUR DIGESTED SLUDGE SAMPLES, METROPOLITAN
SYRACUSE TREATMENT PLANT.

Fraction from Differential Centrifugation*	Recovered Radioactivity in Seeded, 5-Day Anaerobic Sludge Samples			
	8/19 (70.7g/L)	10/13 (54.8g/L)	12/1 (61.0g/L)	12/ 29 (N.D.)
1. <u>Soluble</u>	702	1214	693	1298
2. <u>Colloidal</u>	778	2849	1035	2152
3. <u>Supra- colloidal</u>	4831	6084	4212	4958
4. <u>Settleable</u>	1314	1154	1742	672

- * 1. Resists pelleting at 62,500 x g, 15 min
2. Pellets at 62,500 x g, 15 min
3. Pellets at 4,800 x g, 30 min
4. Pellets at 1 x g, 2 hr

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TABLE 10. REPRODUCIBILITY OF SUCROSE DENSITY GRADIENT FRACTIONATION OF VIRUS-CONTAINING SLUDGE SOLIDS FROM THE ANAEROBIC DIGESTER TANKS.^{b,c}

Differential Centrifuga- tion	Sucrose Fraction	Radioactivity Recovered ^a						$\bar{x}$	σ
		1	2	3	4	5	6		
<u>Supra- colloidal</u> (Pellets at 4,800 x g)	3A	1575	1451	1448	1515	1578	1572	1523	61.7
	3B	461	473	510	548	469	515	496	33.9
	3C	968	1357	1164	1201	1146	1181	1170	124.5
	3D	1805	1802	1538	1716	1433	1575	1645	152.7
		4809	5083	4660	4980	4625	4810	4831	198.7
<u>Settleable</u> (Pellets at 1 x g)	4A	459	358	411	414	323	426	407	36.6
	4B	406	327	380	494	572	470	442	88.0
	4C	312	715	306	430	459	500	453	150.2
	4D	89	119	97	137	102	84	105	20.0
		1266	1519	1194	1085	1506	1480	1314	192.3

^aDisintegrations per 10 minutes (¹²⁵I) corrected for background.

^bMetropolitan Syracuse Treatment Plant

^c ¹²⁵I-poliovirus 1 was mixed with 80-100 mL samples of anaerobic digester sludge in 125 mL screw-cap Erlenmeyer flasks with magnetic stirring bars. The mixture was incubated anaerobically at 30°C for 5 days with stirring. The caps were loosened periodically to valve off gas. The samples were fractionated first by differential centrifugation and then by sucrose gradient fractionation. The pellets from 4,800 x g and 1 x g settling were diluted 1:5 and placed on sucrose gradients from 25 to 65% sucrose.

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during centrifugation at 4,800 x g. The settleable fraction apparently fragments very readily after being resuspended and mixed by pipetting up and down several times with a 5-mL pipette.

ADSORPTION OF VIRUSES BY SPECIFIC MICROBES: BACTERIA, ALGAE AND CILIATES

Adsorption of poliovirus 1 by Zoogloea ramigera

A common if not universal component of zoogloae in trickling filters is Z. ramigera. It is also responsible for zoogloea bulking in activated sludge and has been isolated frequently from the flocs of "normal" sludge. Microscopic examination of these flocs reveals both intracellular granules and extracellular granular masses which are Sudanophilic. These granules have been isolated and analyzed as PHB. Cells producing the granules floc very quickly; non-producers remain dispersed. The adsorptive properties of Z. ramigera for virus particles are illustrated in Table 11. Four fractions from an iodinated poliovirus 1 preparation were mixed with Z. ramigera for either 1 or 17 hrs. Total DPM for the sample was determined, the cells were pelleted at low speed and half the supernatant was removed and counted for residual activity (data in Table 11 are corrected for the sample size). Some of the cells had been given supplemental glucose for 10 hrs to increase PHB accumulation.

The experiment shows clearly that Z. ramigera was a strong adsorber. The adsorption was rapid and complete since no additional counts were removed in 17 hrs of gentle shaking-adsorption. No significant differences were observed between high titers of virus (fraction A) and low titers (Fraction B). In this instance the addition of glucose had no effect on the adhesiveness.

The PHB granules were efficient adsorbents by themselves (Table 12). Addition of the ¹²⁵I-labelled virus to undegraded, native PHB granules resulted in large losses of virus label, 48 to 94 percent. The PFU titer decreased 99.0 to 99.92 percent.

The adsorption of poliovirus by PHB is not unique to this virus. Phage f2 was adsorbed equally effectively by PHB granules. Table 13 shows the effect of virion numbers on the relative binding of f2 by the same number of PHB granules (0.5 mg/ml). A 6 log difference in titer between the highest and lowest virus preparations revealed less than one log difference in the percent adsorption. One must conclude that we did not begin to saturate the virus-binding sites in this PHB preparation. It is interesting to find a wastewater-related surface which will bind f2, since this phage is being considered as a possible standard for measuring the effectiveness of virus disinfection procedures (75) and its loss to adsorption would be a necessary factor to consider.

Relationship of Zoogloea ramigera to primary virus removal

The previous experimental period showed that ¹²⁵I-poliovirus 1 was adsorbed quickly by both the mixed liquor suspended solids (MLSS) and

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solids from the primary digester. This observation was extended to include other virus groups (Table 14). There appears to be little difference between the viruses tested. Reovirus was low in titer and labeling so the low count is probably not significant. These data are in agreement with many kinetic studies of adsorption by a variety of suspended biological organic solids, indicating that 95 percent of most viruses are adsorbed within one hour in the presence of cations. These include EMC virus (39), poliovirus 1 (76, 77) coxsackievirus A9 (76) and phage f2 (78).

Adsorption of poliovirus appeared to be a common property of zoogloal flocs in a glucose-arginine growth medium. In one experiment, after a 4 hour adsorption period strain ZR 1-16-M removed 94 percent of the ¹²⁵I-poliovirus label; ZR 115, 98 percent; ZR 106, 97 percent; ZR 216, 89 percent. Cell densities were approximately 2 to 5 x 10⁸ cells/ml.

The importance of divalent and trivalent cations in adsorption of viruses is illustrated in Table 15. Two different types of Z. ramigera cultures were used--ZR 115, which forms "fingerlike projections" as a result of capsular production (79), and ZR 1-16-M, a non-slime producer (which flocs nevertheless) (80). Both organisms adsorbed poliovirus 1 in the presence of all cations, with the exception of calcium which inhibited adsorption. Trivalent ions and magnesium were most effective. Washed cells without cations adsorbed very little poliovirus. This corresponds closely to the experiences of many investigators using selective filter techniques to concentrate viruses from rat water and sewage. Aluminum and magnesium are required for isolation of the viruses on membrane filters and as a result reversible metal complexes are formed between the virions and the filter surface and between inorganic flocs and the viruses (31). The concentrations of ions can be very critical for adsorption from certain types of water (64). Iron complexes have been used recently to remove viruses from suspension where the organic content is not too high (64,81).

Zoogloea flocs do not emulate membrane filters in every respect. Maximum adsorption for polioviruses on filters is usually under acid conditions with the addition of aluminum ion and the pH maintained at pH 3.5 with buffer. When Zoogloea were stripped of miscellaneous ions with EDTA and then replated with aluminum at three concentrations (pH 3.5), the polio virus was bound with equal effectiveness over the pH range of 5 to 8. The isoelectric point of the aluminum complex is about 5.5 and a strong binding is in evidence there. The controls which had been stripped with EDTA continued to bind some virus, suggesting that adherence is not entirely dependent on a cation bridge between the virus and the cell floc.

Trapping and encapsulation of virions by microbial flocs

Although it is clear that viruses are adsorbed to bacterial flocs, it is not known whether or not the virions are trapped inside the cell aggregates as they form. This encapsulization may explain in part the difficulties encountered in trying to recover viruses from sludge solids. It is particularly relevant if conditioning processes, ie., addition of CaCl₂ or NaCl, are applied to sludge and cause flocs to come apart.

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TABLE 11. ADSORPTION OF RADIOIODINATED POLIOVIRUS 1 BY ZOOGLOEA
RAMIGERA CELLS GROWN SIX HOURS IN A LOW CARBON-NITROGEN MEDIUM.⁶

0.5 Percent Glucose Supplement	Polio-1 Fraction **	Adsorption Period *	Total DPM in Sample +	DPM in Super- natant +	Percent Adsorbed
+	1A	1 hr	1104	182	84
	1B		878	388	56
	2A		409	52	87
	2B		388	78	80
-	1A	1 hr	1396	161	88
	1B		633	182	71
	2A		333	32	90
	2B		302	310	0
+	1A	17 hr	1538	414	73
	1B		2673	654	75
	2A		711	190	73
	2B		380	134	65
-	1A	17 hr	2558	638	75
	1B		757	420	45
	2A		675	150	78
	2B		515	188	64

** Poliovirus from sucrose gradient: 1A = 25% sucrose; 1B = 23%;
2A = 21%; 2B = 19%.

* 30 C with continuous agitation

+ Corrected for background

⁶ Glucose (0.5 percent) was added to half the cultures 10 hrs be-
fore addition of the ¹²⁵I-poliovirus. Virus was adsorbed either
1 hr or 17 hr before the cells were pelleted at 10,000 x g, 10
min and DPM in the supernatant were determined.

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TABLE 12. ADSORPTION OF RADIOIODINATED
POLIOVIRUS 1 BY POLYHYDROXYBUTYRATE
GRANULES.*

Polio-1 Fraction	Total DPM in Sample ⁺	DPM in Super- natant ⁺	Percent Adsorbed (x 100)
1A	1807	106	94
1B	681	234	66
2A	392	104	73
2B	473	246	48

* Adsorption period: one hour at 30°C with
continuous agitation, concentration:
0.5 mg PHE/mL.

⁺ Corrected for background.

TABLE 13. ADSORPTION OF PHAGE f2 BY PHB
GRANULES.*

PFU/ml in Adsorption Tube	PFU/ml in Supernatant	Percent Adsorption
2.6×10^{11}	1.56×10^9	94.0
2.6×10^9	2.09×10^8	92.0
2.6×10^7	6.7×10^5	97.4
2.6×10^5	2.5×10^3	99.0
2.6×10^5 (no PHB)	2.4×10^5	-

* Adsorption period: 1 hr, 30°C with
continuous agitation.

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TABLE 14* ADSORPTION OF RADIOIODINATED VIRUSES BY ZOOGLEA
RAMIGERA CELLS GROWN SIX HOURS IN A LOW CARBON-NITROGEN MEDIUM.†

Virus	DPM x 10			PFU ⁷ (x 10 ⁷)
	Total	Remaining in supernatant	Percent Adsorption	
Adenovirus 7	4,991	799	84	2.4
Adenovirus 31	8,663	865	90	8
Echovirus 7	15,430	618	96	80
Coxsackievirus B3	14,390	719	95	60
Reovirus 1	815	236	71	0.5

* Z. ramigera I-16-M

† Glucose (0.5 percent) was added to half the culture 1 hr before addition of the viruses. Adsorption period: 1 hr; adsorption tube: 2 mL with 2×10^7 cells/mL.

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TABLE 15. ADSORPTION OF ¹²⁵I-POLIOVIRUS 1 BY ZOOGLAEA RAMIGERA CELL FLOCS
WITH METAL IONS (0.001M, pH 5.6).

Ion	<u>Z. ramigera</u> 115 cell floc			<u>Z. ramigera</u> I-16-M cell floc		
	¹²⁵ I-polio Added	¹²⁵ I-polio Unadsorbed	Percent Removed	¹²⁵ I-polio Added	¹²⁵ I-polio Unadsorbed	Percent Removed
Al ⁺³	6593	370	94	3704	98	97
Ca ⁺²	"	6124	7	"	3003	19
Cd ⁺²	"	4780	28	"	1824	51
Cu ⁺²	"	4125	37	ND	ND	ND
Fe ⁺³	"	1388	79	3704	806	78
Hg ⁺²	"	3264	50	"	2171	41
Mg ⁺²	"	942	86	"	1367	63
Pb ⁺²	"	1113	83	"	915	75
Zn ⁺²	"	5381	18	"	2426	35
None	"	6075	13	"	2963	20

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Zoogloea ramigera 115 was grown on the surface of 200 μ m microcarrier beads until the beads were uniformly covered with cells. The cultures were shifted to high glucose for 2 hrs, ^{125} I-poliovirus was adsorbed to the surface and the unadsorbed virus was removed by washing. The beads were then incubated 10 to 12 hrs in high glucose after various treatments: (1) addition of fresh, dispersed cells (2) addition of dispersed cells to cell-coated beads sterilized with chloroform-saturated H_2O or 50 percent EtOH; (3) no treatment; original coating was allowed to multiply. Finally ^3H -labelled virus was adsorbed to the doubly coated beads as a surface marker. The coated beads were "unpeeled" by sequential washings with NaCl (4 mg/ml) and distilled H_2O . First washings removed most of the ^3H -labelled virus in all treatments and small amounts of ^{125} I-label from beads (1) and (3). The ^{125} I internal marker on killed cells was difficult to strip off. The data are interpreted as showing that most of the viruses adsorbed to glyco-calyx of the first cells remain in the "core" of the floc. The small amount of virus that appeared on the surface may be moved in the "finger-like projections of the colonies which are typical of Zoogloea. Thus, removal of the virus requires degradation of the capsule.

Quantitation of virus-adsorbing bacteria in sludge flocs

Activated sludge flocs were disaggregated by placing washed aliquots of settled sludge in shaker culture with 0.1 percent arginine and 0.1 percent yeast extract, (C/N = 5:1), pH 7.4 for 6 to 12 hrs. The cells were sonicated lightly, washed and allowed to settle in polystyrene petri plates. The unattached cells were removed by washing with saline- $CaCl_2$ and the attached cells overlaid with a medium containing 0.1 percent proteose peptone, 0.1 percent yeast extract, 1 percent glucose, vitamin B_{12} , biotin and 0.8 percent agar (C/N = 10:1). The colonies which developed were picked and subcultured in microtiter wells in C/N = 5:1 overnight. Glucose, biotin and B_{12} were added to the culture and a 2 mm glass bead was placed in each well. After cells had attached to the beads, the coated beads were handled with forceps for washing and immersion in ^{125} I-labelled poliovirus 1 in glucose for various periods of time. Unadsorbed virus was removed by washing and the residual radioactivity was determined. Significant virus adsorption was observed in 39/389 isolates from four sludge samples (Table 16).

Oxygen dependence of the removal process

A technique devised by C. P. Gerba to elute enteroviruses from water filtrates was used in an attempt to differentiate between the rapid, short term removal and the slow, long term removal of viruses by activated sludge. The solids were pelleted at 10,000 x g, 20 minutes and the pellet was eluted at pH 11.5 (Table 17). Desorption at pH 11.5 was effective in artificially seeded sludge for at least 60 minutes, but by 4 hours 99.9% of the virions were refractory even though free virus was still in the supernatant. By 27 hrs after adsorption, few PFU could be demonstrated either in the supernatant or were eluted from the pellet. When aeration was reduced by halting and shaking, addition of NaCN or bubbling N_2 through the seeded sludge sample, the adsorption was greatly reduced. The seed

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TABLE 16. ADSORPTION OF ^{125}I -POLIOVIRUS TO BEADS COATED WITH PURE CULTURES OF BACTERIA FROM ACTIVATED SLUDGE FLOCS. *,†

Percent of virus adsorbed on beads	Range (Percent)					
	1-10	11-20	21-30	31-40	41-50	51-60
No. of positive wells	389	2	1	0	3	1
					10	16
					6	0

*100 percent = $9.86 \pm 0.53 \times 10^3$ DPM

†uncoated beads = $1.6 \pm 0.3 \times 10^2$ DPM

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TABLE 17. OXYGEN DEPENDENT DECAY OF VIRUSES IN MLSS
(2900 mg/L) AFTER SEEDING WITH COMMERCIAL TRIVALENT
POLIOVIRUS VACCINE.

Treatment	Adsorption Period (hrs)	Recovery from Sediment (PFU/ml)	
		Free Virus	Elution at pH 11.5 ^{*,+}
-	0	2.1×10^5	2.1×10^5
Shaking	1	1.5×10^5	1.9×10^5
	4	3.0×10^5	6.3×10^2
	27	7	0
Stationary	1	1.8×10^5	1.8×10^5
	4	2.0×10^5	7.9×10^4
	27	4.2×10^3	3.0×10^3
4 x 10 ⁻³ M NaCN, Stationary	1	1.8×10^5	1.6×10^5
	4	1.0×10^5	8.6×10^4
	27	1.0×10^5	9.1×10^4
Anaerobic	1	2.3×10^5	1.2×10^5
	4	2.4×10^5	6.7×10^4
	27	1.0×10^5	5.1×10^4

*Elution with 0.25M glycine, pH 11.5, 0.01 M
EDTA, blender 5 min. (C. Gerba, personal
communication).

⁺pH was adjusted to 7.2 after blending.

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virus in the supernatant was only slowly reduced and proportionally, more of the seed which was adsorbed could be eluted at pH 11.5. While not conclusive, the data suggest that the permanent removal of virus by activated sludge has an oxygen dependent, cyanide sensitive component.

Kelly et al. (82) demonstrated that aeration of the sludge floc was necessary to get virus removal after starter sludge was used as an inoculum and that malonic acid or anaerobiosis interfered with the removal of poliovirus by the sludge. Clarke et al. (76) showed that removal of virus was roughly proportional to the biomass generated and hence a strongly aerobic process. They failed to remove significantly greater quantities by adjusting the pH value to 4.9, 6.5, 7.2 and 8.5; extraction with diethyl ether and 1:5,000 versene were equally ineffective. Clarke and Chang (83) similarly failed to dissociate poliovirus 1, echovirus 12 and coxsackie-virus A12 from trickling filter "slime" at pH 8.0-8.3 in a blender.

Transfer of ¹²⁵I-poliovirus label to protozoa

Radiolabelled virus adsorbed to MLSS began an irreversible disappearance about 30 to 100 minutes after its initial adsorption (58). Tables 18 and 19 describe experiments designed to test the possibility that virions adsorbed to zoogloal flocs may be consumed by ciliated protozoans seen grazing on the flocs (83) and associated with the quality of the effluent (84, 85). Actively dividing ciliate cultures were fed both zoogloal floc and PHB granules with adsorbed poliovirus. After 12 hours the protozoa were killed with a few drops of methanol, removed by low speed centrifugation, washed 2X and assayed for radioactivity. The data (Table 18a) suggest that significant amounts of virus label were in fact transferred to Colpidium and Euplotes by the cell floc and by the PHB granules. This activity does not remain permanently with the protozoa. After 5 days growth the protozoa were fixed with glutaraldehyde, washed with PBS and counted for residual activity. No significant ¹²⁵I was detected in the cells.

The low activity transferred in the previous experiment was overcome by adapting protozoan populations to feeding on Z. ramigera (Table 18b). Colpidium and Paramecium caudatum were able to grow with Zoogloea as the nutrient source; P. aurelia grew on a mixed culture that was predominantly Zoogloea but also contained an Aerobacter-like organism. As a consequence, adsorbed ¹²⁵I-poliovirus was taken up strongly by Colpidium and P. caudatum and only slightly by P. aurelia. Both granules and cells were effective in the transfer. Wild ciliate isolates from aeration tank flocs showed a limited capacity to take up virus. It was not clear how many of these cells were viable, active feeders during the 5 day exposure. The uptake of ZR 115 polymer-poliovirus was less than that of the PHB granules. Nevertheless, the uptake seems a significant indicator that virus can be transferred to another species via this route. Colpidium cells were washed 2X with sterile cereophyll medium and refed with unlabeled Zoogloea. At the end of 5 days the protozoa were assayed and found to be unlabeled. The label was largely adherent to the culture tube walls. P. caudatum can cause the flocculation of India ink particles and other particles as a

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TABLE 18A. REMOVAL OF RADIOIODINATED, ADSORBED POLIOVIRUS 1 BY CILIATED PROTOZOANS 12 HRS AFTER FEEDING BACTERIA OR GRANULES.[⊙]

Species	125I-poliovirus adsorbed to <u>Z. ramigera</u> cells ⁺			125I-poliovirus adsorbed to native PHE granules		
	Pellet (DPM)	Supern. (DPM)	Removal (%)	Pellet (DPM)	Supern. (DPM)	Removal (%)
<u>Paramecium aurelia</u> ^{**}	177	1984	8	210	3862	6
<u>Colpidium</u> spp. [*]	528	1891	24	1346	2008	39
<u>Spirostomium</u> sp. [*]	61	1503	3	29	3453	0
<u>Euplotes</u> sp. [*]	398	1827	18	625	3440	18
Control	9	2020	-	312	4217	-

^{**} Monoxenic culture

^{*} Mixed culture, predominant organism

⁺ Z. ramigera 1-16-M: 3.6×10^6 cells in 2 mL cerophyll medium plus 0.5 percent yeast extract.

[⊙] Protozoa centrifuged 2X at 240 x g, 10 min.

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TABLE 183. ROLE OF CILIATED PROTOZOANS IN THE REMOVAL OF ^{125}I -POLIOVIRUS ADSORBED TO ZOOGLA PANIGERA AND/OR ITS CONSTITUENTS.

Carrier of ¹²⁵ I-labelled polio-1 virus	Poliovirus 1 ¹²⁵ I-label per tube (DPM x 10)	¹²⁵ I-label associated with protozoa after 5 days (DPM x 10)				
		<u>Colpidium</u>	<u>Paramecium aurelia</u>	<u>Paramecium caudatum</u>	Mixed Ciliates from Activated Sludge MLSS	Control
<u>Z. ramigera</u> I-16-M. grown in low glucose	7,997	423(5%)	0	692(9%)	21	0
<u>Z. ramigera</u> I-16-M. grown in high glucose	"	1301(16%)	184	5,006(62%)	892	86
<u>Z. ramigera</u> 115 crude polymer + Al ³⁺	"	865(11%)	ND	ND	ND	212
Polyhydroxybutyrate granules	"	1212(15%)	563	4,846(61%)	ND	14
None	"	50(.06%)	0	327(4%)	93	0
Number of protozoa	-	3,000	3,000	1,200	2,000	0
Culture constituents	-	<u>Z. ramigera</u> I-16-M + yeast powder, glucose, pep- tone	Aero- bacter + Cerophyll	Aero- bacter + Cerophyll	Cerophyll + Mixed sludge bacteria	Cero- phyll

• Meadowbrook-Limestone Treatment Facility

result of substance "P", a mucoprotein it secretes which facilitates ingestion. This may explain in part the high avidity shown by P. caudatum in this experiment. Whether or not Colpidium produces such a mucoprotein is unknown.

Role of ciliates in disposal of adsorbed virions

Tetrahymena thermophila (T. pyriformis B-3) was easily adapted to Z. ramigera 115. Consumption of ZR115 by Tetrahymena was influenced by the type of bacterial growth medium and the length of time the cells were grown in glucose. High levels of glucose and/or 17 hr exposure resulted in reduced consumption of ZR115. In some experiments Tetrahymena removed 61 to 95 percent of labeled poliovirus 1 adsorbed to glucose-grown ZR115 (4 to 6 hr prior exposure to glucose). When sterile, filtered sludge supernatants or water extracts of primary sludge were added to the glucose-grown cells the removal was often stimulated. The nature of this factor is unknown.

Uptake of viruses by protozoan grazing

Tetrahymena pyriformis was adapted to Zoogloea ramigera which had adsorbed poliovirus 1 doubly labelled with ^{125}I and ^3H -uridine (Table 19). The ^{125}I : ^3H ratio in Tetrahymena cells was determined after 24 hrs of feeding and the ratios of Tetrahymena-associated counts were determined 24, 48, 72 and 96 hours later. These were, respectively, 0.95, 0.97, 1.05 and 1.10 in a single experiment. Only a 28% reduction in total counts was observed, suggesting that relatively little virus degradation occurs intracellularly.

Adsorption of poliovirus by algae

Photosynthetic microorganisms are probably in contact with viruses in sewage treatment outfalls, perhaps being responsible in part for stream and pond self-purification. Some of these organisms have a high avidity for PV-1 at least. Table 20 shows that blue green algae were generally more effective than green species in removing the virus. The blue-green organisms are characterized by conspicuous capsules related to bacterial exopolysaccharides in some instances. The Chlorotyllium isolate was not bacterial free and it may have been the bacterial epiphytes rather than the alga per se which were the adsorptive surface. It remains to be demonstrated whether or not epiphytic microbes on filamentous algae function in this manner in nature to purify contaminated water.

INTERACTION OF VIRUSES WITH MICROBIAL EXOPOLYSACCHARIDES AND PHB GRANULES

Adsorption of ^{125}I -polioviruses by native PHB granules

Some strains of Z. ramigera such as I-16-M and 115 produce such vast quantities of PHB when grown on high carbohydrate media that the cells literally burst, releasing the particles with membranes intact (66, 86).

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TABLE 19. STABILITY OF ^{125}I -POLIOVIRUS REMOVED BY
TETRAHYMENA PYRIFORMIS GRAZING ON ZOOGLAEA RAMIGERA.

Hours after ^{125}I - and ^3H -polio feeding on viruses	Isotope	DPM	Ratio I/H	Normalized Ratio
24	$\frac{^{125}\text{I}}{^3\text{H}}$	$\frac{8831}{7639}$	1.16	1.0
48	$\frac{^{125}\text{I}}{^3\text{H}}$	$\frac{8010}{7215}$	1.11	0.95
72	$\frac{^{125}\text{I}}{^3\text{H}}$	$\frac{7203}{6540}$	1.12	0.97
96	$\frac{^{125}\text{I}}{^3\text{H}}$	$\frac{6547}{5366}$	1.22	1.05
120	$\frac{^{125}\text{I}}{^3\text{H}}$	$\frac{6294}{4955}$	1.27	1.10

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TABLE 20. ADSORPTION OF PV-1 BY ALGAL POLLUTANTS
TREATED WITH 0.005M METAL IONS, 3 HR, pH 5 IN MODI-
FIED BRISTOL'S SOLUTION, 22°C.*

Alga	Percent adsorption of PV-1 label								
	None	Al	Cd	Cu	Hg	Mg	Ni	Pb	Zn
Gleotrichia	27	89	77	20	66	80	9	63	24
Nostoc 586	38	98	99	44	77	89	23	51	13
Nostoc 31	40	90	98	45	41	91	26	58	15
Oscillatoria	5	27	19	20	12	8	4	50	26
Ulothrix	4	18	8	ND	ND	36	9	13	18
Chlamydomonas	0	2	12	ND	ND	24	25	26	2
Chlorella	1	11	5	ND	ND	13	20	29	5
Chlorotyllum	23	87	64	ND	ND	27	22	13	0

*Two mL of 5 percent w/v treated cells were washed 3 times with water by centrifugation. Two mL ¹²⁵I-PV-1 inoculum were added to the algal pellet; adsorption was 1 hr, 22°C. After adsorption the algal cells were pelleted by centrifugation at 2,500 x g, 15 minutes, and 1 mL of the supernatant fluid was removed and counted. PV-1 inoculum was approximately 1 x 10⁸ PFU with 4,176 DPM/2mL.

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TABLE 21. REMOVAL OF ^{125}I -POLIOVIRUS
ADSORBED TO NATIVE PHB GRANULES.

Treatment	Pellet (DPM x 10)	Supern. (DPM x 10)	Percent ^{125}I Released
Phosphate-saline, pH 7.2	9,304	180	2
0.25 percent trypsin	5,262	3,498	60
0.25 percent trypsin + 0.01M EDTA	5,337	3,786	58
0.1 percent pronase	7,061	2,318	75
Ethyl ether extraction, 20°C, 15 min	7,119	1,777	20
Chloroform: ether extraction (1:3)	6,759	2,786	29
Alkaline hypochlorite*	757	8,464	92

*Griebal, R.J. and J.M. Merrick. J. Bacteriol. 108, 782, 1971.

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However, other reports question whether or not the level of carbohydrate needed to induce this phenomenon ever occurs in waste waters (87, 88).

An attempt was made to examine whether or not PHB granules could provide an identifiable adsorptive surface in wastewater flocs. There is no convincing evidence that microbial enzymes in sludge normally release viruses from PHB granules or any other surface; solvent extraction of sludge does not increase virus recovery unless it is accompanied by sonication. Thus it appears from Table 21 that while native PHB granules bind polioviruses, the release of virus from the granules is not typical of sludge-adsorbed virus. Poliovirus 1 was released by proteinases and by solvents; alkaline hypochlorite, which denatures PHB but does not remove isotope-labelled virus from sludge, released most of the ^{125}I . Thus it would appear that the poliovirus in sludge solids is not associated with PHB trapped in the exogenous capsules.

Viruses and *Z. ramigera* exopolymers

Since PHB granules are trapped in the exocellular matrix of *Z. ramigera* and these were demonstrated to adsorb poliovirus and f2 phage avidly, it is not certain whether the adherence of viruses to the cell floc is related to the exocellular polymers or to the surface of the PHB granules. Purified ZR 115 and ZR I-16-M exocellular polymers were extracted, washed with 0.05 M EDTA and replated with cations at pH 5.5. Virus was then adsorbed to the polymer at pH 6.5. Table 22 demonstrated that adsorption of viruses by the cell-free exopolymer had many of the characteristics of the *Z. ramigera* cell floc. Aluminum, iron, and magnesium provided maximum adherence in a 2-hr adsorption period. There was a striking correspondence between the adsorption of virus by the floc and the polymer. This is also seen in Table 23 which was an attempt to recover adsorbed virus from the exocellular polymer. Again adsorption was promoted by Al^{3+} , Fe^{3+} , and Mg^{2+} to 94.5, 91.3 and 77.5 percent, respectively; the washed control adsorption was 28.8 percent.

The reversible nature of this adsorption was demonstrated by removal of the ^{125}I under alkaline conditions (Table 23). The virus-adsorbed polymer was extracted twice with 0.05 M glycine buffer, pH 11.5 for 3 minutes and pelleted by centrifugation at 8,000 x g; the supernatants were combined, neutralized and counted. Aluminum complexes and iron complexes are soluble above pH 9 (31). Release of the virus from the polymer is consistent with the alkaline extraction of sewage solids, raw water precipitates and estuarine sediments reported by several laboratories.

Adsorption to sewage solids polymers

Extraction of microbial slime polymers was performed by the method of Dugan (69) using concentrated treatment plant solids as a source (Table 24). The dried polymer extracted this way was a moderately effective poliovirus adsorbent, especially in the presence of aluminum ion. This lends more credence to the proposition that enterovirus removed during activated sludge treatment may be related to *Zoogloea*-type polymers.

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TABLE 22. ADSORPTION OF ^{125}I -POLIOVIRUS 1 BY ZOOGLOEA RAMIGERA EXOCELLULAR POLYMERS TREATED WITH METAL IONS.^a

Ion ^o	<u>Z. ramigera</u> 115 polymer ^a			<u>Z. ramigera</u> I-16-M polymer ^b		
	^{125}I -polio added	^{125}I -polio undisorbed ^d	Percent removed	^{125}I -polio added	^{125}I -polio undisorbed	Percent removed
Al ⁺³	6481	134	98	3777	412	89
Ca ⁺²	"	6003	7	"	3285	13
Cd ⁺²	"	5245	19	"	2796	26
Cu ⁺²	"	4796	26	ND	ND	ND
Fe ⁺³	"	391	94	3777	1148	70
Hg ⁺²	"	3301	49	"	1469	61
Mg ⁺²	"	1104 ^f	83	"	941 ^f	75
Ni ⁺²	"	6529	0	ND	ND	ND
Pb ⁺²	"	898	86	3777	945	72
Zn ⁺²	"	4666	28	"	3351	11
None ^e	"	5759	11	"	3263	14

^a0.15 g dry wt in 20 mL

^b0.12 g dry wt in 20 mL

^c0.01 M metal as the chloride used to wash the polymer 4 hrs and washed twice with double distilled water

^dDisintegrations per 10 minutes

^eWashed with 0.05 M EDTA, pH 6.5, 4 hrs

^fVisible precipitate

^gAbsorption period = 2 hrs

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TABLE 23. RECOVERY OF ^{125}I -POLIOVIRUS 1 FROM ZOOGLAEA RAMIGERA
I-16-M EXOCELLULAR POLYMER TREATED WITH VARIOUS CATIONS.

Adsorbed ion	Treatment			
	pH 11.5	pH 11.5, 0.05M EDTA	pH 9.0, 3% beef extract	
Al^{3+}	DPM adsorbed **	7911	7742	7019
	DPM released *	8230	7740	5830
	% DPM released	104	100	84
Fe^{3+}	DPM adsorbed **	6396	7505	8002
	DPM released *	6290	7320	7570
	% DPM released	98	98	95
Mg^{2+}	DPM adsorbed **	6269	6832	5840
	DPM released *	6130	6020	5270
	% DPM released	98	88	90
None	DPM adsorbed **	2637	2043	2233
	DPM released *	2550	2160	1990
	% DPM released	97	106	90

** Initial activity added to each tube was 7996/10 min.

* Corrected for dilution.

+ Adsorption at pH 5.5.

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TABLE 24. ADSORPTION OF POLIOVIRUS BY CRUDE
POLYMER EXTRACTS OF SEWAGE SOLIDS.^①

Solids	Removal of ¹²⁵ I-poliovirus**	
	Crude extract	Crude extract + 0.001M Al ³⁺
Aeration tank, suspended solids* 0.23g/20 mL	3758 (59%)	4447 (69%)
Raw sludge* 0.10 g/20 mL	837 (13%)	2339 (36%)
Primary digester ⁺ sludge 0.31 g/20 mL	2577 (40%)	3273 (72%)

** ¹²⁵I-poliovirus 1 = 6435 DPM x 10

* Meadowbrook-Limestone Plant

+ Metropolitan Syracuse Plant

① Data are DPM x 10 associated with the high
speed pellet; arenttheses show percent of
original activity unadsorbed.

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Relationship of glycocalyx to virus adhesion and shock loading of activated sludge

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Sludge solids were partially fractionated in an Imhoff cone by settling for 6 hr at 23°C. The supernatant was removed by suction as well as the upper third of the settled solids which contained mostly small aggregates of cells and relatively few protozoans. The solids in this fraction were washed three times with distilled water by low speed centrifugation and resuspended in (1) 1 percent peptone plus 0.1 percent yeast extract, (2) distilled H₂O, or (3) filtered supernatant which had been removed from the solids in the Imhoff cone. After aerated shaking for 24 hr at 30°C, cells in peptone-yeast extract were largely disaggregated and did not attach strongly to the bottoms of microtiter wells or to microscope slides. Addition of 0.1 percent glucose caused immediate adherence of many bacteria; most could not be removed by vigorous washing and other treatments. Clones of adhering and nonadhering bacteria were isolated and tested for PV-1 and f2 adsorption. Data are still preliminary but it appears that PV-1 is absorbed much more strongly by adherent clones than by non-adherent isolates. Numerous gram negative, urealytic pseudomonad-like bacteria appear to be common.

Quantitative removal of ¹²⁵I-labeled virus was achieved by coating tubes with adherent bacteria. Glucose, salts and virus were added to washed, peptone-grown cells in a roller tube culture for 4 to 12 hr, unattached cells and virus were decanted, the cells were washed and the tubes were counted in an automated gamma counter. This system permits evaluation of variables such as pH, metal ion concentration, temperature, detergents, etc. Since the glycocalyx is probably mucopolysaccharide we were especially interested in studying the interference of virus adsorption with sugars, amino sugars, amino acids, fatty acids, etc. In other systems, such as the phage-bacterium interaction, the kinetics of adsorption have been evaluated in this fashion. However, a wide spectrum of low molecular weight substances has consistently failed to show any specific interference with virus adsorption to the cells or exopolysaccharides.

RECOVERY OF ADSORBED VIRUSES

Desorption of ¹²⁵I-poliovirus from PHB and *Z. ramigera*

The interaction of viruses with PHB and *Z. ramigera* may serve as a useful test system to improve the methods for recovering viruses. A variety of published methods to desorb viruses from sewage solids, filters, cell debris, etc. were applied to this test system after virus adsorption for 1 hr. (Table 25). Initial adsorption was 77 percent of the ¹²⁵I label by PHB and 80 percent by *Z. ramigera*. Elution with the eight eluants produced very little increase in unadsorbed label. The PFU eluted in the experiment varied from 0 to 40 percent of the applied virus and showed very little duplication within the same replicate samples. The tenacity of the binding sites in these experiments reflected almost perfectly the experiences of investigators attempting to elute virus from sludges after longer adsorption periods.

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TABLE 25. DESORPTION AND RECOVERY OF RADIOIODINATED POLIOVIRUS 1 FROM PHB GRANULES AND Z. RAMIGERA.*

No.	Elutant	Percent ¹²⁵ I-poliovirus 1 released by treatment with elutant	
		PHB	<u>Z. ramigera</u>
1.	0.05M glycine, pH 11.5, 5 min, 30°C	25	36
2.	0.25M glycine, pH 11.5, 0.01M EDTA	25	29
3.	Sonication, 90 seconds, 20 KV	31	16
4.	4 cycles freeze-thawing	33	29
5.	0.01% Tween 80, 60 min, 30°C	29	36
6.	10% fetal calf serum in PBS, pH 7.4	34	27
7.	3% beef extract, pH 9.0	27	26
8.	Tryptose phosphate broth	31	12

* Adsorption period: 60 minutes, 30°C.

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TABLE 26. DESORPTION OF PPU FROM SLUDGE SOLIDS.

Sludge Source	Sludge Type	Date	PPU eluted from washed sludge *			
			solids pH 11.5, 0.05 M glycine		solids pH 7.5, 0.01M Al ₃ plus	
			Adsorbed	Eluted	Adsorbed	Eluted
Onondaga Metro- politan	anaerobic digester solids	1-16-78	1.88x10 ⁷	1.6x10 ⁷	1.6x10 ⁷	1.7x10 ⁷
		2-13-78	1.69x10 ⁷	1.6x10 ⁷	1.9x10 ⁷	2.0x10 ⁷
		2-20-78	1.8x10 ⁷	1.5x10 ⁷	1.9x10 ⁷	5.9x10 ⁶
		3-10-78	1.9x10 ⁷	1.1x10 ⁷	1.8x10 ⁷	1.3x10 ⁷
Limestone- Meadowbrook	aerobic activated sludge	1-16-78	1.3x10 ⁷	1.1x10 ⁷	1.8x10 ⁷	1.6x10 ⁷
		2-13-78	1.4x10 ⁷	1.1x10 ⁷	8.0x10 ⁶	7.3x10 ⁶
		3-10-78	1.5x10 ⁷	1.3x10 ⁷	2.6x10 ⁷	3.6x10 ⁶
Baldwins- ville	aerobic activated	4-4-78	1.4x10 ⁷	1.1x10 ⁷	1.9x10 ⁷	1.8x10 ⁷

* 1.88x10⁷ PPU were added to 30 mL 5 percent sludge solids.

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The almost uncanny duplication of the counts released—regardless of the type of treatments—and the extreme variability of the PFU released suggest that the recoveries of "free virus" result from comminution of the virus aggregates during pipetting and other manipulations.

ROLE OF METALS AND pH IN VIRUS ADHERENCE TO SLUDGE SOLIDS

Interactions between primary aerobic sludge digest and ¹²⁵I-labeled polio-virus 1

Although previous study had shown that aluminum, magnesium and iron ions increased adsorption of poliovirus 1 (PV-1) and other enteroviruses in pure culture of *Z. ramigera*, the addition of these ions to sludge solids at pH 7.5 improved adsorption very little or not at all (Table 26). Repeated washing of the solids with 0.05M EDTA reduced their adsorptive capacity about 50 percent; addition of 0.01M Al³⁺, pH 5, to the EDTA washed sludge caused the adsorption of an additional log of virus. Desorption of the virus ¹²⁵I-label with glycine buffer, pH 11.5, was reasonably constant at 59 to 80 percent recovery; viability counts varied for unknown reasons in the eluted samples.

However, later experiments showed that the pH 11 stripping of the sludge was greatly reduced in efficiency as the contact time between the virus and the sludge solids increased. In Table 26 a 10 to 15 minute contact time gave good recoveries at pH 11; in Table 25 a contact time 60 minutes resulted in only slightly improved yields.

Dried sludge from the Metropolitan Plant anaerobic digester was used as a virus adsorbent (Table 27). Difficulties in resuspending these solids made it impossible to quantitate the removal of PV-1 and phages f2 and Ø174 very accurately, but some virus removal was effected with Al³⁺, pH 3.5. A search is being made for an appropriate wetting agent. Dried filamentous algae including 4 green and 4 blue-green algae which grow in polluted water were examined for their ability to adsorb PV-1 after being washed with the following metal salts: nickel, mercury, lead, zinc, copper, cadmium, aluminum and magnesium (Table 20). The adsorption of PV-1 was unequivocally increased by di- and trivalent ions. We have data for these algae which show that large quantities of several of these metals are absorbed from ordinary nutrient solutions and deposited as the metal hydroxides on the exterior surfaces of the cells.

The effect of metal hydroxide complexes is dramatically clear in Table 28. The relative adsorption and release of ¹²⁵I-labeled PV-1 from flocs of a gram negative sewage isolate (now believed to be *Klebsiella* type 3) is shown. The alkaline unstable aluminum complex was hydrolyzed and released the PV-1 at higher pH values. Although metallic ions are contributory to adherence, their hydroxide complexes do not cause irreversible adsorption after 60 minute exposure.

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TABLE 27. ADSORPTION OF ^{125}I -LABELED VIRUSES BY DRIED ANAEROBIC DIGESTER SLUDGE,
PH 3.5, 0.005M AlCl_3 .

Percent (w/v) solids	Experiment No.	PV-1			f2		
		DPM Added *	DPM Remaining Unadsorbed *	Percent Adsorbed	DPM Added *	DPM Remaining Unadsorbed *	Percent Adsorbed
2	1	8,651	5,146	41	6,008	1,372	77
	2	4,325	3,819	12	3,004	1,867	38
	3	4,325	1,514	65	3,004	1,805	39
	4	4,325	2,842	66	3,004	1,067	64
	5	4,325	3,075	29	3,004	1,211	37
5	1	8,651	5,398	38	6,008	4,846	19
	2	4,325	1,730	60	3,004	902	70
	3	4,325	2,651	61	3,004	1,114	63
10	1	8,651	2,556	70	6,008	855	86
	2	4,325	543	88	3,004	1,953	65
	3	4,325	1,602	63	3,004	628	79

* Corrected for background.

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TABLE 28. ALUMINUM/pH INTERACTION ON THE SURFACE FILM OF AN ADHERENT
PSEUDOMONAD, 78-004.

Event	pH	Aluminum chloride				
		0:1M	0.05M	0.005M	0.0001M	0
Adsorption	3	99.99%	99.995%	99.99%	91.3%	24%
	4			99.995%		
	5			99.0%		
	6			37.0%		
	7			25.0%		
Elution	8			1.0%		
	9			10.0%		
	10			63.0%		
	10.5	88.0%	101.0%	109.0%	88.0%	90.0%
	11	94.0%		94.0%		100%

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SECTION 6

GENERAL DISCUSSION AND CONCLUSIONS

It is clear from our work that some portion of the microbial population in activated sludge can produce exocellular polymers (glycocalyx) with a high avidity for nonenveloped viruses. Perhaps the only reason why 10 percent or more of wild viruses remain unadsorbed during activated sludge treatment is that we deliberately discourage formation of polymer slimes as a cause of unwanted sludge bulking. In addition, many laboratory tests purporting to determine virus removal by sludge are carried out under conditions where the sludge is provided low carbohydrate levels and high nitrogen levels, the least efficient means of synthesizing copious quantities of microcalyx on the bacterial cells.

The research addressed itself to a need for understanding both the biological and biophysical factors which contribute to the "adsorption loss" of virus particles to water-borne solids. It is also concerned with the development of new methods for assaying *in situ* the viruses adsorbed to such particulates. Some experimental evidence suggests that even when adsorbed to fecal solids and other colloids, viruses often are infectious and should be assayed when we examine the sedimentable solids of water supplies for microbial pathogens. Adsorption may even increase infectivity by concentrating multiple virions on particles which can be retained at sensitive sites in the host (92). If we understand the processes involved in virus adsorption and can locate and determine the number of "insoluble" virions, we will have the theoretical basis for making a systematic search for techniques to desorb the natural spectrum of wild viruses associated with fecal solids and sewage colloids. Additionally, with this knowledge we should be in a position to recommend procedures for improving the removal of viruses by activated sludge, i.e., accelerating the adsorption rates, improving the avidity of viruses and sewage colloids, and promoting viricidal activities of sludge microbes in the sewage floc.

PROPERTIES OF MICROBIAL FLOCS IN ACTIVATED SLUDGE TREATMENT

It is sometimes overlooked that sewage organisms are not treatment organisms. In fact coliforms are removed very quickly to become part of the soluble nutrient for growing a captive biomass more-or-less anchored due to its adhesiveness. In the activated sludge process, typically with a 30 percent return of solids and a 6 hour detention time, the floc is overfed during the early part of the detention period and accumulates intracellular and extracellular masses. This material is variously reported as PHB, polysaccharide, volutin, lipopolysaccharide, and sulfur globules (97).

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Many of these granules are enclosed in charged membranes and it may be the membranes rather than the inclusion materials which are the binding sites for nonencapsulated viruses.

The polycationic nature of the floc results in the adsorption of not only microorganisms and subcellular debris, but also heavy metals, which are avidly removed. Anionic colloids attached to these surfaces become in themselves an adsorptive surface.

The organisms responsible for the adhesive flocs are not well defined. In trickling filters the dominant populations have long doubling times; in activated sludge they have shorter doubling times. Floc formation has been attributed to pseudomonads, Bacillus sp., Sphaerotilus natans, and Z. ramigera as well as filamentous fungi. Fifty or more species of organisms have been isolated from activated sludge flocs, many under the description of Zoogloea sp. (98, 59). Not all colonies of Z. ramigera cause floc formation of zoogloea (87), but all genera of floc-forming bacteria--Sphaerotilus, Bacillus, Pseudomonas and Zoogloea--form PHB under shock loads of soluble carbohydrate (99).

Table 29 illustrates the absorptive properties of selected bacteria from primary activated sludge. A sludge sample was incubated in peptone broth for 6 hours at 30°C with aeration and then diluted in 0.5 percent glucose salts. Approximately 400 colony-forming units were placed in polystyrene plates and incubated for 3 hrs, 30°C. The supernatant with unattached cells was mixed with penassay broth; the attached cells were washed gently with PBS and overlaid with penassay broth. Plates were incubated at 30° and 37°C and a battery of isolates was obtained. These cells were measured for their ability to (1) attach to polystyrene and (2) to adsorb ¹²⁵I-PV-1. In general, there was a high correlation between the ability of an isolate to attach to polystyrene and to adsorb viruses in the presence of aluminum ion. Ruthenium red staining (93) was also a fairly good predictor of this ability as well.

The kinetics of attachment of bacteria to polystyrene and other surfaces have been reviewed recently (89, 112, 113). Fletcher has summarized accurately what is probably occurring during cell sorption to plastic and perhaps when viruses are adsorbed nonspecifically to microcalyx of bacteria:

"...There is always the tendency for surfaces to obtain a minimum free energy by satisfying the potential bonding capacity of the surface and one way this can be done is through the adsorption of substances (for example, bacterial surface polymers) onto the surface. Thus adsorption of components onto surfaces is favored by a reduction in the free energy of the system and is determined by whether the two molecular phases involved possess common forces (for example, dispersion, dipole) and thus can interact specifically." (89)

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TABLE 29. COMPARISON OF VIRUS ADSORBING CAPACITY OF SLUDGE BACTERIA ISOLATED BY GLUCOSE-INDUCED ADHERENCE AFTER GROWTH IN PEPTONE MEDIUM.

Clone No.	Adherence to polystyrene after addition of glucose/salts	Gram Stain	Morphology	PHB granules	Flagella	Adsorption PV-1 in presence of Al ₃₊ **
78-001	+	-	short rod	+	polar	>90%
78-002	+	-	short rod	+	polar	>90%
78-004	+	-	short rod	+	single, polar	>90%
78-005	+	+	streptototacillus	-	-	60%
78-006	+	+	diplococcus, micrococcus	+	-	>90%
78-050	-	+	sporulating bacillus	ND	-	0
78-051	-	-	diplococcus, micrococcus	ND	-	< 10%
78-052	-	+	yeast	ND	-	0
78-053	-	-	short rod	ND	peritrichous	30%
78-064	-	-	short rod	ND	peritrichous	60%
78-065	-	+	sporulating bacillus	ND	-	5%

** Peptone grown cells were placed in roller tubes containing Earle's basal salts solution, 0.5% glucose, 0.05% yeast extract. After 12 hr the nonadherent cells were decanted, the cell layers were washed with H₂O and with 0.005M AlCl₃. I₂5I-PV-1 in 0.5 ml 0.01M glycine buffer, pH 5, was added and the roller tubes were rotated 10 to 30 times/hr for 2 hr. Unadsorbed virus was removed by 5x rinses with glycine buffer; washed tubes with residual I₂5I were assayed directly.

* PHB granules were produced extracellularly.

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Polyhydroxybutyrate granules

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The data reconfirm earlier observations that PHB granules are very sticky and avid virus adsorbents. Treatment of the native granules with proteinases markedly reduced adsorption; treatment of adsorbed viruses under non-degradative conditions caused the release of viruses. It appears that the adsorption sites are on the proteinaceous PHB granule membrane. Whether or not cations are involved is unknown at this time. Magara and Nambu (100) believe that aluminum sulfate precipitation of activated sludge flocs depends on the presence of PHB or its derivatives on the surface of the cells. Nevertheless, treatments which release ¹²⁵I from PHB granules--proteinases, peptidases, chloroform extraction, alkaline hypochlorite--do not resemble treatments which release polioviruses from sewage solids. Furthermore, Z. ramigera strains which produce few stainable extracellular granules (ZR 106) may adsorb viruses strongly.

On the basis of these and other observations, it is concluded that PHB granules could not be the main adsorbing site except under very unusual conditions, viz., a high carbohydrate shock load such as fruit canning waste. Nevertheless, large amounts of PHB are found in sucrose gradient fractions with particles 0.1-0.3 μ m diameter. Similar fractions are reputed to adsorb much of the virus in raw sewage (94, 95) and sea water (96).

By using PHB granules, dispersed cells of Z. ramigera and floc cells of Z. ramigera as the elements for a test system, it should be possible to study the kinetics of virus adsorption and to make predictions concerning the efficiency of virus removal by sludge. It may be profitable to determine the efficacy of viricidal materials or processes in this model and to study loss (or gain) in infectivity of virions as a result of adsorption. The reputed high degree of stability of the virus-PHB granule may be a significant contributing factor to the level of the infectious dose. Further, granules may act like an adjuvant for the immune system (92). It has been reported that some commercial spray emulsifiers enhanced experimental encephalomyocarditis virus infections of L929 cell cultures, caused "Reye's-like syndrome" in mice, and rendered L929 cells unable to respond to interferon (82, 93). Emulsifiers such as these, (blends of dodecylbenzene sulfonate and polyoxyethylene ethers), have surface properties somewhat reminiscent of lipopolysaccharides or PHB granules. The possibility of the latter being a vector needs to be tested more rigorously since significant numbers of viruses have been found to be associated with 0.3 μ m diameter particles.

DIFFERENTIAL CENTRIFUGATION OF SLUDGE SOLIDS AND IDENTIFICATION OF VIRUS BINDING SITES

Even under the most ideal conditions, total virus recovery from sludge is rarely possible. Several degradative factors in addition to adsorption will remove a portion of the viruses which were added experimentally to sewage; ammonia (104), temperature (102), microbial enzymes (103), heavy metals (105), aggregate formation (106), osmotic shock, etc. Thus some

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virus radiolabel from degraded virions would be expected to occur in fractions other than those associated with cell flocs or cell polymers. Only rather small amounts of label were observed in the upper gradient layers away from the expected fractions. Very little infectivity was found in the upper fractions. The size and origins of these materials remain to be demonstrated more precisely. Double labeling of the viruses with ^3H -uridine as well as ^{125}I would help determine those gradient fractions receiving degraded virions. It would also assist in identifying artifacts such as self-iodinated lactoperoxidase contaminating the virus preparations.

A somewhat similar study has been published recently by T. W. Hejkal et al. (94) using (1) combinations of rotor speed and/or flow rate to fractionate treatment plant solids and (2) sonication to homogenize the samples before assay. Virus associated with solids larger than $0.3\ \mu\text{m}$ was reduced from 28 percent in the influent to 3.4 percent in unchlorinated effluent. The $0.3\ \mu\text{m}$ particles probably correspond to our fraction B in the sucrose gradients (Table 10). Our B fraction contained quantities of "cell debris" but few whole cells.

EXOCYLLULAR POLYMERS

The clear cut adsorption of viruses by zoogloal polymers makes this organism and its pseudomonad near relatives appear as candidates for real biological events. The adsorption of viruses by the zoogloal flocs corresponds to Z. ramigera's capacity to synthesize polymer, to adhere to polystyrene and glass, to coalesce from small flocs to big flocs, etc. (107). Floc formation and adherence are common phenomena among marine psueodmonads and it would be of interest to determine whether their exocellular polymers also adsorb viruses in a nonspecific fashion. Strains of Klebsiella isolated from sludge and raw sewage in this study are also avid adsorbents for PV-1 (J. Killory, unpublished data).

The principal difficulty in formulating a cohesive theory concerning the role of zoogloal polymers and adsorption is related in part to nomenclature difficulties. The Zoogloea cultures described have widely variable physiological characteristics and their taxonomy is unspecified. Especially troublesome is the fact the biochemistry of their polymers is uncertain (69, 108). Preservation of some of the early isolates has not been possible and some of the previous studies of their surface properties will need to be repeated.

Recently it was found that the isolation of Zoogloea polymers can be accomplished more easily and directly by the method of Choy and Dutton (90) involving a simple extraction of cells with 1 percent phenol followed by precipitation with ethanol and with Cetavlon. Exopolysaccharides prepared in this fashion can precipitate 99.995 percent of enteroviruses from suspension in the presence of 0.005M Al^{+3} .

ROLE OF CATIONS IN ZOOGLOEAL FLOCS

Zoogloea evidences a natural association between its polymers and

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metal ions. For example, iron is deposited around the flocs when iron salts are incorporated into growth media (109) and extensive amounts of copper, cobalt, nickel, zinc and other metals have been demonstrated to form strong complexes (69) with both purified polymer and cell flocs. Thus it appears that zoogloea are very well equipped to bind water-borne viruses through metal complexing and by manipulation of pH, total alkalinity, soluble organics, nutrient supplies, etc.

It is rarely possible to assign a single role to any component affecting sorption of small particles such as viruses to large particles such as flocs. Nevertheless, at the level of cations commonly used (viz., $Al^{3+} = 0.005M$) (Table 30), their first function appears to be a destabilization of the colloidal floc structure as a result of charge neutralization. During this initial interaction the particles are too far apart for the flocculant ions to bridge the surfaces. This is probably the phase of "reversible sorption" (91). At various times it has been suggested that this initial interaction undergoes a rapid reversal as the distance between interacting surfaces decreases and physical forces rather than electrostatic ones become predominant. Recent studies with proteins have revealed the existence of numerous non-ionic "domains" on or near the surface of the molecules in TMV and phage T4 heads which interact through a combination of forces, effecting a balance between the double-layer boundary and van der Waals forces, the DLVO theory (40). The sorption surface of flocs is also a dynamic one where metal hydroxides are continuously deposited, hydrolyzed and replaced (along with carbonates and phosphates). Thus a changing array of potentially reactive sites is always available in the floc to interact with the nucleocapsid of the virus particles.

ELUTION RECOVERY OF SEED VIRUS FROM SEWAGE SOLIDS AND ZOOGLOEA POLYMERS

Our recovery of live adsorbed viruses has been largely dependent upon solubilizing the aluminum complex formed between virus capsids and membrane filters. Favored methods for doing this have been (1) raising the pH to 11.5, (2) raising the pH to 11.5 in the presence of EDTA, (3) raising the pH to 9 with the addition of fetal calf serum (FCS) and (4) addition of 3 percent beef extract. The methods were devised empirically and the explanations for their effectiveness (or lack of it) are only slowly emerging: the virus-adsorbing aluminum hydroxide complexes formed maximally at pH 3.5 to 5.5 are insoluble below pH 9, hence pH 11.5 serves to remove the aluminum ions from the absorbent. Inclusion of EDTA may assist this and also remove other adsorbed cations or prevent them from occupying the sites vacated by Al^{3+} . At pH 9, other metal-binding complexes, such as magnesium, are more readily solubilized and FCS or its hydrated albumin can compete with the virus proteins for attachment sites. The effect of beef extract remains enigmatic but intuitively seems related to the other three methods.

Data show that our modified method for recovering virus from crude sewage solids was effective (pH 11.5 extraction followed by Al^{3+} precipitation at pH 5.5, solubilization with EDTA and detoxification by passage through a one-step sucrose gradient) only when the adsorption period was short term, i.e., 10 minutes. Long adsorption periods, i.e. 1 to 5 hours,

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TABLE 30. INFLUENCE OF pH ON THE ADSORPTION OF
¹²⁵I-POLIOVIRUS 1 BY ZOOGLOEA RAMIGERA I-16-M CELL
FLOCS AFTER COATING WITH ALUMINUM ION. *

Aluminum M	pH	¹²⁵ I-polio Added	¹²⁵ I-PV-1 Unadsorbed	Percent PV-1 Adsorbed
.05	5.0	3421	210	94
.005		"	58	98
.0005		"	82	98
none		"	3092	10
.05	6.0	"	274	92
.005		"	69	98
.0005		"	96	97
none		"	2297	33
.05	7.0	"	628	82
.005		"	342	90
.0005		"	164	95
none		"	2099	39
.05	8.0	"	490	86
.005		"	535	83
.0005		"	684	80
none		"	2899	15

* Adsorption flasks contained 0.76 g dry wt. cells in 20 mL. Cells were prewashed with 0.05M EDTA, pH 6.5, 4 hrs. Adsorption period: 2 hrs.

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"fixed" the virions to the surface so that the complex could not be readily hydrolyzed. Interestingly, the recovery range of 23 to 65 percent is close to that reported by others (81, 108, 110) for resuspension of polio-virus-aluminum complexes eluted from membrane filters.

ROLE OF PROTOZOA IN VIRUS REMOVAL

Ciliated protozoa have been identified as the dominant protozoa present in activated sludge and their numbers are associated with the quality of the effluent (85, 97, 111). As the principal grazing population, they are likely to consume much of the adsorbed materials in sludge, including viruses. Paramecium caudatum and probably other ciliates secrete a sticky polysaccharide ("P" substance) that flocculates bacteria and nonbiological particles such as India ink (98). The ink particles can be ingested after being coated. Although this study has just begun to follow the ingestion of adsorbed viruses by protozoa, these preliminary data already suggest this will prove to be a common phenomenon.

The demonstrated capacity of virus to be transferred to cultured protozoans as well as wild ciliates opens up new and exciting possibilities for vector transmission, virus alteration, and kinetic studies of virus removal. Certainly critical studies must be made of the virus types which can be ingested and their survival rate. Numerous questions such as the longevity of viruses relative to the protozoan life cycle will occur. Is the virus actually ingested or merely adsorbed to "P" substance, the pellicle, etc.? The cytology and possibly the cytopathology of the host recipients will be intriguing. It appears that at least the exterior coat of the virus is excreted and the need for more sophisticated labeling of virus components arises. And finally the question which originated the study, are protozoa actually responsible for the irreversible loss of viruses during waste treatment? What percentage of the 1×10^4 PFU/L commonly found in sewage are ingested by the ciliates?

FATE OF VIRUSES IN SEWAGE TREATMENT AND IN EFFLUENT

The incoming virus population arrives at the primary aeration tanks in a great variety of ways, principally in a state of passive adsorption rather than as free particles. Their adsorbers include bacterial capsules which have been synthesized by gut organisms; cells of bacteria, fungi, ciliates and algae; insect casts, egg cases, eggs, and fecal pellets; paper and vegetable fiber; hair and skin; emulsions of fats, lipids, road oil hydrocarbons, industrial lubricants, and detergents; and inorganic particles. Despite their interaction with carrier surfaces the virions are surrendered during the activation process, possibly as the flocs develop on the surface of the sewage particles and degrade the outer surfaces. The mechanism of the transfer of virions to floc can only be conjectured, but seems likely that some viruses are captured, others released. As the floc density increases, free viruses may be captured with an efficiency not unlike that of phages and specific hosts.

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The exopolysaccharide (XPS) capsules neutralize and "inactivate" un-enveloped viruses through several mechanisms. During aeration the capsules of dead and dying bacteria are stripped away. Such dispersed XPS can coat virions, apparently blocking their receptors and even precipitating or agglutinating them. It binds them to inanimate objects which may settle out with sludge. In the laboratory XPS will bind PV-1 and phage to glass surfaces; in holding ponds it probably binds them to water plants and algae. The XPS also may reappear in a dispersed form as part of the undigested bacterial walls excreted by grazing ciliates and amoebae. For the same reason they may be associated with the microfecal pellets of nematodes and other grazing fauna.

Although unencapsulated viruses can be demonstrated to adsorb to PHB granules, volutin, pili, and flagellae, the survivability of these organelles during activation seems problematical. What these materials contribute to nonsedimentable solids in the final effluent is an interesting but unmeasured element at present.

Thus one arrives at the speculative conclusion that the initial 0.1 to 10 percent of the sewage viruses which end up in the effluent arrive there as their bacterial carrier eventually die or is consumed and the relatively resistant capsular material washes out with the overflow. It predicts that these particles will be characterized by: (1) traces of XPS associated with some virions; (2) altered antigenicity of their surface; (3) a non-specific "stickiness"; (4) clumping of virions; (5) heavy metals adsorbed to the XPS; (6) reactivity to certain lectins and to anti-XPS antibody; (7) altered electrophoretic mobility; and (8) generally a reduced capacity to infect sensitive cells.

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